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# METHODS FOR PRODUCING BIOLOGICAL SUBSTANCES IN ENZYME-DEFICIENT MUTANTS OF *ASPERGILLUS NIGER*

## Cross-Reference to Related Application

This application claims the benefit of U.S. Provisional Application No. 60/459,902, filed March 31, 2003, which application is incorporated herein by reference.

## Background of the Invention

### Field of the Invention

The present invention relates to methods of producing heterologous biological substances in enzyme-deficient *Aspergillus niger* mutant strains, methods of obtaining the enzyme-deficient *Aspergillus niger* mutant strains, and the enzyme-deficient *Aspergillus niger* mutant strains.

### Description of the Related Art

*Aspergillus niger* secretes large quantities of glucoamylase. However, *Aspergillus niger* hosts with the desirable traits of increased protein expression and secretion may not necessarily have the most desirable characteristics for successful fermentation. The fermentation may not be optimal because of the secretion of multiple enzymes requiring removal during the recovery and purification of a biological substance of interest or the enzymes may co-purify with the biological substance.

Boel *et al.*, 1984, *EMBO J.* 3: 1097-1102, 1581-1585, disclose the cloning of the glucoamylase (*glaA*) gene of *Aspergillus niger*. Fowler *et al.*, 1990, *Curr. Genet.* 18: 537-545 disclose the deletion of the glucoamylase (*glaA*) gene of *Aspergillus niger*.

Korman *et al.*, 1990, *Curr. Genet.* 17: 203-217 disclose the cloning, characterization, and expression of two alpha-amylase genes (*amyA and amyB*) from *Aspergillus niger* var. *awamori*. U.S. Patent No. 5,252,726 discloses the cloning of two full length neutral alpha-amylase genes from *Aspergillus niger*.

U.S. Patent No. 5,252,726 discloses the cloning of a portion of an acid stable alpha-amylase gene (*asa*) from *Aspergillus niger*.

Pedersen *et al.*, 2000, *Metabolic Engineering* 2: 34-41, and WO 00/50576 disclose the disruption of an oxaloacetate hydrolase (*oah*) gene encoding oxaloacetate hydrolase (EC 3.7.1.1) in a glucoamylase-producing strain of *Aspergillus niger*, wherein the resulting strain was incapable of producing oxalic acid.

WO 01/68864 discloses that *prtT*-disrupted *Aspergillus niger* strains are protease deficient, indicating that deletion of *prtT* expression in a host strain can result in an increase in the level of recoverable protein susceptible to proteolysis.

It is an object of the present invention to provide improved *Aspergillus niger* hosts which combine the capacity for expression of commercial quantities of a biological substance while being deficient in the production of enzymes which can complicate recovery and downstream processing of the biological substance of interest.

## Summary of the Invention

The present invention relates to methods of producing a heterologous biological substance, comprising:

(a) cultivating a mutant of a parent *Aspergillus niger* strain in a medium suitable for the production of the heterologous biological substance, wherein (i) the mutant strain comprises a first nucleotide sequence encoding the heterologous biological substance and one or more second nucleotide sequences comprising a modification of *glaA* and at least one of the genes selected from the group consisting of *asa*, *amyA*, *amyB*, *prtT*, and *oah*, and (ii) the mutant strain is deficient in the production of glucoamylase and at least one enzyme selected from the group consisting of acid stable alpha-amylase, neutral alpha-amylase A, and neutral alpha-amylase B, protease, and oxalic acid hydrolase compared to the parent *Aspergillus niger* strain when cultivated under identical conditions; and

(b) recovering the heterologous biological substance from the cultivation medium.

The present invention also relates to enzyme-deficient *Aspergillus niger* mutant strains and methods for producing the enzyme-deficient *Aspergillus niger* mutant strains.

## Brief Description of the Figures

Figure 1 shows a restriction map of pJRoy10.

Figure 2 shows a restriction map of pMBin01+.

Figure 3 shows a restriction map of pJRoy17.

Figure 4 shows a restriction map of pSMO127.

Figure 5 shows a restriction map of pMBin05.

Figure 6 shows a restriction map of pMBin04+.

Figure 7 shows a restriction map of pMBin09.

Figure 8 shows a restriction map of pMBin10.

Figure 9 shows a restriction map of pMBin02.

Figure 10 shows a restriction map of pMBin03.

Figure 11 shows a restriction map of pMBin08.

Figure 12 shows the effect of the *prtT* deletion on protease activity.

Figure 13 shows the effect of the *prtT* deletion on *Candida antarctica* lipase B activity.

Figure 14 shows a comparison of *Scytalidium thermophilum* catalase production in *Aspergillus niger* general host strains MBin114, MBin118 and MBin120.

## Detailed Description of the Invention

The present invention relates to methods of producing a heterologous biological substance, comprising: (a) cultivating a mutant of a parent *Aspergillus niger* strain in a medium suitable for the production of the heterologous biological substance, wherein (i) the mutant strain comprises a first nucleotide sequence encoding the heterologous biological substance and one or more second nucleotide sequences comprising a modification of *glaA* and at least one of the genes selected from the group consisting of *asa*, *amyA*, *amyB*, *prtT*, and *oah*, and (ii) the mutant strain is deficient in the production of glucoamylase and at least one enzyme selected from the group consisting of acid stable alpha-amylase, neutral alpha-amylase A, and neutral alpha-amylase B, protease, and oxalic acid hydrolase compared to the parent *Aspergillus niger* strain when cultivated under identical conditions; and (b) recovering the heterologous biological substance from the cultivation medium.

An advantage of the present invention is the elimination or reduction of glucoamylase and at least one enzyme selected from the group consisting of acid stable alpha-amylase, neutral alpha-amylase A, and neutral alpha-amylase B, protease, and oxalic acid hydrolase in an *Aspergillus niger* fermentation broth simplifies downstream processing of heterologous biological substances.

The term "amyloglucosidase" is defined herein as a dextrin 6-alpha-D-glucanohydrolase activity which catalyses the endohydrolysis of 1,6-alpha-D-glucoside linkages at points of branching in chains of 1,4-linked alpha-D-glucose residues and terminal 1,4-linked alpha-D-

glucose residues. For purposes of the present invention, glucoamylase activity is determined according to the procedure described by Fagershom and Kalkkinen, 1995, *Biotechnol. Appl. Biochem.* 21: 223-231, where the glucose produced by a glucoamylase from 0.1 M maltotriose is measured using a glucose oxidase assay kit (Sigma Chemical Co., St. Louis, MO) at pH 4, 25°C. One unit of glucoamylase activity is defined as 1.0 μmole of glucose produced per minute at 25°C, pH 4.

The term "alpha-amylase activity" is defined herein as a 1,4-alpha-D-glucan glucanohydrolase activity which catalyzes the endohydrolysis of polysaccharides with three or more alpha-1,4-linked glucose units in the presence of water to maltooligosaccharides.

The term "acid stable alpha-amylase activity" is defined herein as an alpha-amylase activity with optimal activity in the acid pH range. For purposes of the present invention, acid stable alpha-amylase activity is determined using 4,6-ethylidene (G7)-p-nitrophenyl (G1)-alpha-D-maltoheptaside as substrate using Sigma Chemical Co. Kit 577 at pH 4.0.

The term "neutral alpha-amylase activity" is defined herein as an alpha-amylase activity with optimal activity in the neutral pH range. For purposes of the present invention, neutral alpha-amylase activity is determined using 4,6-ethylidene (G7)-p-nitrophenyl (G1)-alpha-D-maltoheptaside as substrate using Sigma Chemical Co. Kit 577 at pH 7.0.

The term "oxalic acid hydrolase" is defined herein as an enzyme activity which catalyzes the conversion of oxaloacetate in the presence of water to oxalic acid and acetate. The enzyme is classified as belonging to EC 3.7.1.1. For purposes of the present invention, oxaloacetate hydrolase activity is determined according to the procedure described in the Examples section herein. One unit of oxaloacetate hydrolase activity is defined as 1.0 μmole of oxalic acid produced per minute at 30°C, pH 7.5.

The term "modification" is defined herein as an introduction, substitution, or removal of one or more nucleotides in a gene or a regulatory element required for the transcription or translation thereof, as well as a gene disruption, gene conversion, gene deletion, or random or specific mutagenesis of *glaA* and at least one of the genes selected from the group consisting of *asa, amyA, amyB, prtT*, and *oah*. The deletion of the *glaA* gene and *asa, amyA, amyB, prtT*, and/or *oah* gene(s) may be partial or complete. The modification results in a decrease or elimination in expression of *glaA* and at least one of the genes selected from the group consisting of *asa, amyA, amyB, prtT*, and *oah*.

In a preferred aspect, the modification results in the inactivation of *glaA* and at least one of the genes selected from the group consisting of *asa, amyA, amyB, prtT*, and *oah*. In another

preferred aspect, the modification results in a decrease in expression of *glaA* and at least one of the genes selected from the group consisting of *asa, amyA, amyB*, *prtT*, and *oah*. In another preferred aspect, the modification results in the expression of *glaA* and at least one of the genes selected from the group consisting of *asa, amyA, amyB*, *prtT*, and *oah* being decreased, eliminated, or a combination thereof.

In a preferred aspect, the mutant comprises a modification of *glaA* and *asa*. In another preferred aspect, the mutant comprises a modification of *glaA* and *amyA*. In another preferred aspect, the mutant comprises a modification of *glaA* and *amyB*. In another preferred aspect, the mutant comprises a modification of *glaA* and *prtT*. In another preferred aspect, the mutant comprises a modification of *glaA* and *oah*.

In another preferred aspect, the mutant comprises a modification of *glaA*, *asa*, and *amyA*. In another preferred aspect, the mutant comprises a modification of *glaA*, *asa*, and *amyB*. In another preferred aspect, the mutant comprises a modification of *glaA*, *asa*, and *prtT*. In another preferred aspect, the mutant comprises a modification of *glaA*, *asa*, and *oah*. In another preferred aspect, the mutant comprises a modification of *glaA*, *amyA,* and *amyB*. In another preferred aspect, the mutant comprises a modification of *glaA*, *amyA,* and *prtT*. In another preferred aspect, the mutant comprises a modification of *glaA*, *amyA,* and *oah*. In another preferred aspect, the mutant comprises a modification of *glaA*, *amyB*, and *prtT*. In another preferred aspect, the mutant comprises a modification of *glaA*, *amyB*, and *oah*. In another preferred aspect, the mutant comprises a modification of *glaA*, *prtT*, and *oah*.

In another preferred aspect, the mutant comprises a modification of *glaA*, *asa*, *amyA,* and *amyB*. In another preferred aspect, the mutant comprises a modification of *glaA*, *asa*, *amyB*, and *prtT*. In another preferred aspect, the mutant comprises a modification of *glaA*, *asa*, *prtT*, and *oah*. In another preferred aspect, the mutant comprises a modification of *glaA*, *asa*, *amyA,* and *prtT*. In another preferred aspect, the mutant comprises a modification of *glaA*, *asa*, *amyA,* and *oah*. In another preferred aspect, the mutant comprises a modification of *glaA*, *amyA*, *amyB*, and *prtT*. In another preferred aspect, the mutant comprises a modification of *glaA*, *asa*, *amyB*, and *oah*. In another preferred aspect, the mutant comprises a modification of *glaA*, *amyA, prtT*, and *oah*. In another preferred aspect, the mutant comprises a modification of *glaA*, *amyA, amyB*, and *oah*. In another preferred aspect, the mutant comprises a modification of *glaA*, *amyB*, *prtT*, and *oah*.

In another preferred aspect, the mutant comprises a modification of *glaA*, *asa*, *amyA, amyB*, and *prtT*. In another preferred aspect, the mutant comprises a modification of *glaA*, *asa*, *amyB*, *prtT*, and *oah*. In another preferred aspect, the mutant comprises a modification of *glaA*,

*amyA, amyB, prtT*, and *oah*. In another preferred aspect, the mutant comprises a modification of *glaA*, *asa*, *amyA, amyB*, and *oah*. In another preferred aspect, the mutant comprises a modification of *glaA*, *asa*, *amyA*, *prtT*, and *oah*.

In another preferred aspect, the mutant comprises a modification of *glaA*, *asa*, *amyA, amyB*, *prtT*, and *oah*.

The term "deficient" is defined herein as an *Aspergillus niger* mutant strain which produces no detectable glucoamylase and at least one enzyme selected from the group consisting of acid stable alpha-amylase, neutral alpha-amylase A, and neutral alpha-amylase B, protease, and oxalic acid hydrolase compared to the parent *Aspergillus niger* strain when cultivated under identical conditions, or, in the alternative, produces preferably at least 25% less, more preferably at least 50% less, even more preferably at least 75% less, and most preferably at least 95% less glucoamylase and at least one enzyme selected from the group consisting of acid stable alpha-amylase, neutral alpha-amylase A, and neutral alpha-amylase B, protease, and oxalic acid hydrolase compared to the parent *Aspergillus niger* strain when cultivated under identical conditions. The level of enzyme produced by an *Aspergillus niger* mutant strain of the present invention may be determined using methods described herein or known in the art.

In the methods of the present invention, the parent *Aspergillus niger* strain may be a wild-type *Aspergillus niger* strain or a mutant thereof. It will be understood that the term "*Aspergillus niger*" also includes varieties of *Aspergillus niger* (See, for example, Robert A. Samsom and John I. Pitt, editors, *Integration of Modern Taxonomic Methods for Penicillium and Aspergillus Classification*, Harwood Academic Publishers, The Netherlands). In a preferred aspect, the parent *Aspergillus niger* strain is *Aspergillus niger* DSM 12665.

The enzyme-deficient *Aspergillus niger* mutant strain may be constructed by reducing or eliminating expression of *glaA* and at least one of the genes selected from the group consisting of *asa, amyA, amyB, prtT* and *oah* using methods well known in the art, for example, insertions, disruptions, replacements, or deletions. The portion of the gene to be modified or inactivated may be, for example, the coding region or a regulatory element required for expression of the coding region. An example of such a regulatory or control sequence of a gene may be a promoter sequence or a functional part thereof, *i.e.*, a part which is sufficient for affecting expression of the gene. Other control sequences for possible modification include, but are not limited to, a leader, propeptide sequence, signal sequence, transcription terminator, and transcriptional activator.

The *Aspergillus niger* mutant strains may be constructed by gene deletion techniques to

eliminate or reduce the expression of *glaA* and at least one of the genes selected from the group consisting of *asa, amyA, amyB, prtT* and *oah*. Gene deletion techniques enable the partial or complete removal of the gene(s) thereby eliminating their expression. In such methods, the deletion of the gene(s) may be accomplished by homologous recombination using a plasmid that has been constructed to contiguously contain the 5' and 3' regions flanking the gene.

The *Aspergillus niger* mutant strains may also be constructed by introducing, substituting, and/or removing one or more nucleotides in the gene or a regulatory element thereof required for the transcription or translation thereof. For example, nucleotides may be inserted or removed so as to result in the introduction of a stop codon, the removal of the start codon, or a frame-shift of the open reading frame. Such a modification may be accomplished by site-directed mutagenesis or PCR generated mutagenesis in accordance with methods known in the art. See, for example, Botstein and Shortle, 1985, *Science* 229: 4719; Lo *et al.*, 1985, *Proceedings of the National Academy of Sciences USA* 81: 2285; Higuchi *et al.*, 1988, *Nucleic Acids Research* 16: 7351; Shimada, 1996, *Meth. Mol. Biol.* 57: 157; Ho *et al.*, 1989, *Gene* 77: 61; Horton *et al.*, 1989, *Gene* 77: 61; and Sarkar and Sommer, 1990, *BioTechniques* 8: 404.

The *Aspergillus niger* mutant strains may also be constructed by gene disruption techniques by inserting into the gene of interest an integrative plasmid containing a nucleic acid fragment homologous to the gene which will create a duplication of the region of homology and incorporate vector DNA between the duplicated regions. Such gene disruption can eliminate gene expression if the inserted vector separates the promoter of the gene from the coding region or interrupts the coding sequence such that a non-functional gene product results. A disrupting construct may be simply a selectable marker gene accompanied by 5' and 3' regions homologous to the gene. The selectable marker enables identification of transformants containing the disrupted gene.

The *Aspergillus niger* mutant strains may also be constructed by the process of gene conversion (see, for example, Iglesias and Trautner, 1983, *Molecular General Genetics* 189: 73-76). For example, in the gene conversion method, a nucleotide sequence corresponding to the gene(s) is mutagenized *in vitro* to produce a defective nucleotide sequence which is then transformed into the parent *Aspergillus niger* strain to produce a defective gene. By homologous recombination, the defective nucleotide sequence replaces the endogenous gene. It may be desirable that the defective gene or gene fragment also comprises a marker which may be used for selection of transformants containing the defective gene.

The *Aspergillus niger* mutant strains may also be constructed by established anti-sense techniques using a nucleotide sequence complementary to the nucleotide sequence of the gene (Parish and Stoker, 1997, *FEMS Microbiology Letters* 154: 151-157). More specifically, expression of the gene by an *Aspergillus niger* strain may be reduced or eliminated by introducing a nucleotide sequence complementary to the nucleotide sequence of the gene, which may be transcribed in the strain and is capable of hybridizing to the mRNA produced in the strain. Under conditions allowing the complementary anti-sense nucleotide sequence to hybridize to the mRNA, the amount of protein translated is thus reduced or eliminated.

The *Aspergillus niger* mutant strains may be further constructed by random or specific mutagenesis using methods well known in the art, including, but not limited to, chemical mutagenesis (see, for example, Hopwood, *The Isolation of Mutants* in *Methods in Microbiology* (J.R. Norris and D.W. Ribbons, eds.) pp 363-433, Academic Press, New York, 1970) and transposition (see, for example, Youngman *et al.*, 1983, *Proc. Natl. Acad. Sci. USA* 80: 2305-2309). Modification of the gene may be performed by subjecting the parent strain to mutagenesis and screening for mutant strains in which expression of the gene has been reduced or eliminated. The mutagenesis, which may be specific or random, may be performed, for example, by use of a suitable physical or chemical mutagenizing agent, use of a suitable oligonucleotide, or subjecting the DNA sequence to PCR generated mutagenesis. Furthermore, the mutagenesis may be performed by use of any combination of these mutagenizing methods.

Examples of a physical or chemical mutagenizing agent suitable for the present purpose include ultraviolet (UV) irradiation, hydroxylamine, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), N-methyl-N'-nitrosogaunidine (NTG) O-methyl hydroxylamine, nitrous acid, ethyl methane sulphonate (EMS), sodium bisulphite, formic acid, and nucleotide analogues. When such agents are used, the mutagenesis is typically performed by incubating the parent strain to be mutagenized in the presence of the mutagenizing agent of choice under suitable conditions, and selecting for mutants exhibiting reduced or no expression of a gene.

In a preferred aspect, *glaA* comprises a nucleotide sequence having at least 70%, preferably at least 75%, more preferably at least 80%, even more preferably at least 85%, most preferably at least 90%, and even most preferably at least 95% identity to SEQ ID NO: 1. In a most preferred aspect, *glaA* comprises the nucleotide sequence of SEQ ID NO: 1. In another most preferred aspect, *glaA* consists of the nucleotide sequence of SEQ ID NO: 1.

In another preferred aspect, *glaA* comprises a nucleotide sequence which hybridizes under very low stringency conditions, preferably low stringency conditions, more preferably medium stringency conditions, more preferably medium-high stringency conditions, even more

preferably high stringency conditions, and most preferably very high stringency conditions with SEQ ID NO: 1.

In a preferred aspect, *asa* comprises a nucleotide sequence having at least 70%, preferably at least 75%, more preferably at least 80%, even more preferably at least 85%, most preferably at least 90%, and even most preferably at least 95% identity to SEQ ID NO: 3. In a most preferred aspect, *asa* comprises the nucleotide sequence of SEQ ID NO: 3. In another most preferred aspect, *asa* consists of the nucleotide sequence of SEQ ID NO: 3.

In another preferred aspect, *asa* comprises a nucleotide sequence which hybridizes under very low stringency conditions, preferably low stringency conditions, more preferably medium stringency conditions, more preferably medium-high stringency conditions, even more preferably high stringency conditions, and most preferably very high stringency conditions with SEQ ID NO: 3.

In a preferred aspect, *amyA* comprises a nucleotide sequence having at least 70%, preferably at least 75%, more preferably at least 80%, even more preferably at least 85%, most preferably at least 90%, and even most preferably at least 95% identity to SEQ ID NO: 5. In a most preferred aspect, *amyA* comprises the nucleotide sequence of SEQ ID NO: 5. In another most preferred aspect, *amyA* consists of the nucleotide sequence of SEQ ID NO: 5.

In another preferred aspect, *amyA* comprises a nucleotide sequence which hybridizes under very low stringency conditions, preferably low stringency conditions, more preferably medium stringency conditions, more preferably medium-high stringency conditions, even more preferably high stringency conditions, and most preferably very high stringency conditions with SEQ ID NO: 5.

In a preferred aspect, *amyB* comprises a nucleotide sequence having at least 70%, preferably at least 75%, more preferably at least 80%, even more preferably at least 85%, most preferably at least 90%, and even most preferably at least 95% identity to SEQ ID NO: 7. In a most preferred aspect, *amyB* comprises the nucleotide sequence of SEQ ID NO: 7. In another most preferred aspect, *amyB* consists of the nucleotide sequence of SEQ ID NO: 7.

In another preferred aspect, *amyB* comprises a nucleotide sequence which hybridizes under very low stringency conditions, preferably low stringency conditions, more preferably medium stringency conditions, more preferably medium-high stringency conditions, even more preferably high stringency conditions, and most preferably very high stringency conditions with SEQ ID NO: 7.

In a preferred aspect, *oah* comprises a nucleotide sequence having at least 70%, preferably at least 75%, more preferably at least 80%, even more preferably at least 85%, most

preferably at least 90%, and even most preferably at least 95% identity to SEQ ID NO: 9. In a most preferred aspect, *oah* comprises the nucleotide sequence of SEQ ID NO: 9. In another most preferred aspect, *oah* consists of the nucleotide sequence of SEQ ID NO: 9.

In another preferred aspect, *oah* comprises a nucleotide sequence which hybridizes under very low stringency conditions, preferably low stringency conditions, more preferably medium stringency conditions, more preferably medium-high stringency conditions, even more preferably high stringency conditions, and most preferably very high stringency conditions with SEQ ID NO: 9.

In a preferred aspect, *prtT* comprises a nucleotide sequence having at least 70%, preferably at least 75%, more preferably at least 80%, even more preferably at least 85%, most preferably at least 90%, and even most preferably at least 95% identity to SEQ ID NO: 11. In a most preferred aspect, *prtT* comprises the nucleotide sequence of SEQ ID NO: 11. In another most preferred aspect, *prtT* consists of the nucleotide sequence of SEQ ID NO: 11.

In another preferred aspect, *prtT* comprises a nucleotide sequence which hybridizes under very low stringency conditions, preferably low stringency conditions, more preferably medium stringency conditions, more preferably medium-high stringency conditions, even more preferably high stringency conditions, and most preferably very high stringency conditions with SEQ ID NO: 11.

For purposes of the present invention, the degree of identity between two nucleotide sequences is determined by the Wilbur-Lipman method (Wilbur and Lipman, 1983, *Proceedings of the National Academy of Science USA* 80: 726-730) using the LASERGENE™ MEGALIGN™ software (DNASTAR, Inc., Madison, WI) with an identity table and the following multiple alignment parameters: Gap penalty of 10 and gap length penalty of 10. Pairwise alignment parameters are Ktuple=3, gap penalty=3, and windows=20.

The nucleotide sequences disclosed herein or a subsequence thereof, as well as the amino acid sequence thereof or a fragment thereof, may be used to design a nucleic acid probe to identify and clone DNA encoding enzymes involved in the biosynthesis of hyaluronic acid from strains of different genera or species according to methods well known in the art. In particular, such probes can be used for hybridization with the genomic or cDNA of the genus or species of interest, following standard Southern blotting procedures, in order to identify and isolate the corresponding gene therein. Such probes can be considerably shorter than the entire sequence, but should be at least 15, preferably at least 25, and more preferably at least 35 nucleotides in length. Longer probes can also be used. Both DNA and RNA probes can be used. The probes are typically labeled for detecting the corresponding gene (for example, with

$^{32}P$, $^{3}H$, $^{35}S$, biotin, or avidin).

Thus, a genomic DNA or cDNA library prepared from such other organisms may be screened for DNA which hybridizes with the probes described above and which encodes an enzyme in the biosynthetic pathway of hyaluronic acid. Genomic or other DNA from such other organisms may be separated by agarose or polyacrylamide gel electrophoresis, or other separation techniques. DNA from the libraries or the separated DNA may be transferred to and immobilized on nitrocellulose or other suitable carrier material. In order to identify a clone or DNA which is homologous with the nucleotide sequences disclosed herein or subsequences thereof, the carrier material is used in a Southern blot. For purposes of the present invention, hybridization indicates that the nucleic acid sequence hybridizes to a labeled nucleic acid probe corresponding to the nucleotide sequences disclosed herein, its complementary strand, or a subsequence thereof, under very low to very high stringency conditions. Molecules to which the nucleic acid probe hybridizes under these conditions are detected using X-ray film.

For long probes of at least 100 nucleotides in length, very low to very high stringency conditions are defined as prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 μg/ml sheared and denatured salmon sperm DNA, and either 25% formamide for very low and low stringencies, 35% formamide for medium and medium-high stringencies, or 50% formamide for high and very high stringencies, following standard Southern blotting procedures.

For long probes of at least 100 nucleotides in length, the carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS preferably at least at 45°C (very low stringency), more preferably at least at 50°C (low stringency), more preferably at least at 55°C (medium stringency), more preferably at least at 60°C (medium-high stringency), even more preferably at least at 65°C (high stringency), and most preferably at least at 70°C (very high stringency).

For short probes which are about 15 nucleotides to about 70 nucleotides in length, stringency conditions are defined as prehybridization, hybridization, and washing post-hybridization at about 5°C to about 10°C below the calculated $T_m$ using the calculation according to Bolton and McCarthy (1962, *Proceedings of the National Academy of Sciences USA* 48:1390) in 0.9 M NaCl, 0.09 M Tris-HCl pH 7.6, 6 mM EDTA, 0.5% NP-40, 1X Denhardt's solution, 1 mM sodium pyrophosphate, 1 mM sodium monobasic phosphate, 0.1 mM ATP, and 0.2 mg of yeast RNA per ml following standard Southern blotting procedures.

For short probes which are about 15 nucleotides to about 70 nucleotides in length, the carrier material is washed once in 6X SCC plus 0.1% SDS for 15 minutes and twice each for 15 minutes using 6X SSC at 5°C to 10°C below the calculated $T_m$.

A nucleotide sequence homologous or complementary to the nucleotide sequences described herein involved in the production of the enzyme of interest may be used from other microbial sources which produce the enzyme to modify the corresponding gene in the *Aspergillus niger* strain of choice.

In a preferred aspect, the modification of a gene involved in the production of an enzyme in the *Aspergillus niger* mutant strain is unmarked with a selectable marker.

Removal of the selectable marker gene may be accomplished by culturing the mutants on a counter-selection medium. Where the selectable marker gene contains repeats flanking its 5' and 3' ends, the repeats will facilitate the looping out of the selectable marker gene by homologous recombination when the mutant strain is submitted to counter-selection. The selectable marker gene may also be removed by homologous recombination by introducing into the mutant strain a nucleic acid fragment comprising 5' and 3' regions of the defective gene, but lacking the selectable marker gene, followed by selecting on the counter-selection medium. By homologous recombination, the defective gene containing the selectable marker gene is replaced with the nucleic acid fragment lacking the selectable marker gene. Other methods known in the art may also be used.

It will be understood that the methods of the present invention are not limited to a particular order for obtaining the *Aspergillus niger* mutant strain. The modification of a gene involved in the production of an enzyme may be introduced into the parent strain at any step in the construction of the strain for the production of a biological substance. It is preferred that the *Aspergillus niger* mutant strain has already been made enzyme-deficient prior to the introduction of a gene encoding a heterologous biological substance.

In a further aspect of the present invention, the mutants of *Aspergillus niger* strains may contain additional modifications, *e.g.*, deletions or disruptions, of other genes, which may encode substances detrimental to the production, recovery or application of a particular biological substance.

In a preferred aspect, the *Aspergillus niger* strain further comprises a modification, *e.g.*, disruption or deletion, of one or more genes encoding a proteolytic activity. In a more preferred aspect, the proteolytic activity is selected from the group consisting of an aminopeptidase, dipeptidylaminopeptidase, tripeptidylaminopeptidase, carboxypeptidase, aspergillopepsin, serine protease, metalloprotease, cysteine protease, and vacuolar protease.

In another preferred aspect, the *Aspergillus niger* strain further comprises a modification, *e.g.*, disruption or deletion, of one or more genes encoding an enzyme selected from the group consisting of a carbohydrase, carboxypeptidase, catalase, cellulase, chitinase, cutinase,

cyclodextrin glycosyltransferase, deoxyribonuclease, esterase, galactosidase, beta-galactosidase, glucose oxidase, glucosidase, haloperoxidase, hemicellulase, invertase, isomerase, laccase, ligase, lipase, lyase, mannosidase, oxidase, pectinolytic enzyme, peroxidase, phytase, phenoloxidase, polyphenoloxidase, ribonuclease, transferase, alpha-1,6-transglucosidase, alpha-1,6-transglucosidase, transglutaminase, and xylanase.

In the methods of the present invention, the *Aspergillus niger* mutant strain preferably produces at least the same amount of the biological substance as the corresponding parent *Aspergillus niger* strain when cultured under identical production conditions. In a more preferred aspect, the mutant strain produces at least 25% more, preferably at least 50% more, more preferably at least 75% more, and most preferably at least 100% more of the biological substance than the corresponding parent *Aspergillus niger* strain when cultured under identical production conditions.

The *Aspergillus niger* mutant strains are cultivated in a nutrient medium suitable for production of the heterologous biological substance using methods known in the art. For example, the strain may be cultivated by shake flask cultivation, small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermentors performed in a suitable medium and under conditions allowing the biological substance to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or may be prepared according to published compositions (*e.g.*, in catalogues of the American Type Culture Collection). The secreted biological substance can be recovered directly from the medium. If the biological substance is not secreted, it may be obtained from cell lysates.

The biological substances may be detected using methods known in the art that are specific for the biological substances. These detection methods may include use of specific antibodies, high performance liquid chromatography, capillary chromatography, formation of an enzyme product, disappearance of an enzyme substrate, or SDS-PAGE. For example, an enzyme assay may be used to determine the activity of the enzyme. Procedures for determining enzyme activity are known in the art for many enzymes (see, for example, D. Schomburg and M. Salzmann (eds.), *Enzyme Handbook*, Springer-Verlag, New York, 1990).

The resulting biological substance may be isolated by methods known in the art. For example, a polypeptide of interest may be isolated from the cultivation medium by conventional procedures including, but not limited to, centrifugation, filtration, extraction, spray-drying, evaporation, or precipitation. The isolated polypeptide may then be further purified by a variety

of procedures known in the art including, but not limited to, chromatography (*e.g.*, ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (*e.g.*, preparative isoelectric focusing (IEF), differential solubility (*e.g.*, ammonium sulfate precipitation), or extraction (see, *e.g.*, *Protein Purification*, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989). A metabolite of interest may be isolated from a cultivation medium by, for example, extraction, precipitation, or differential solubility, or any method known in the art. The isolated metabolite may then be further purified using methods suitable for metabolites.

The heterologous biological substance may be any biopolymer or metabolite. The biological substance may be encoded by a single gene or a series of genes composing a biosynthetic or metabolic pathway. Thus, the term "first nucleotide sequence encoding the heterologous biological substance" will be understood to encompass one or more genes involved in the production of the biological substance. The term "heterologous biological substance" is defined herein as a biological substance which is not native to the host strain; a native biological substance in which structural modifications have been made to alter the native biological substance, *e.g.*, the protein sequence of a native polypeptide; or a native biological substance whose expression is quantitatively altered as a result of a manipulation of the nucleotide sequence or host strain by recombinant DNA techniques, *e.g.*, a stronger promoter.

In the methods of the present invention, the biopolymer may be any biopolymer. The term "biopolymer" is defined herein as a chain (or polymer) of identical, similar, or dissimilar subunits (monomers). The biopolymer may be, but is not limited to, a nucleic acid, polyamine, polyol, polypeptide (or polyamide), or polysaccharide.

In a preferred aspect, the biopolymer is a polypeptide. The polypeptide may be any polypeptide having a biological activity of interest. The term "polypeptide" is not meant herein to refer to a specific length of the encoded product and, therefore, encompasses peptides, oligopeptides, and proteins. The term "polypeptide" also encompasses two or more polypeptides combined to form the encoded product. Polypeptides also include hybrid polypeptides, which comprise a combination of partial or complete polypeptide sequences obtained from at least two different polypeptides wherein one or more may be heterologous to the *Aspergillus niger* strain. Polypeptides further include naturally occurring allelic and engineered variations of the above-mentioned polypeptides and hybrid polypeptides.

Preferably, the heterologous polypeptide is an antibody, antigen, antimicrobial peptide, enzyme, growth factor, hormone, immunodilator, neurotransmitter, receptor, reporter protein, structural protein, or transcription factor.

In a preferred aspect, the heterologous polypeptide is an oxidoreductase, transferase, hydrolase, lyase, isomerase, or ligase. In a more preferred aspect, the polypeptide is an alpha-glucosidase, aminopeptidase, alpha-amylase, carbohydrase, carboxypeptidase, catalase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, esterase, alpha-galactosidase, beta-galactosidase, glucocerebrosidase, alpha-glucosidase, beta-glucosidase, invertase, laccase, lipase, mannosidase, mutanase, oxidase, pectinolytic enzyme, peroxidase, phospholipase, phytase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transglutaminase, urokinase, or xylanase.

In another preferred aspect, the polypeptide is a collagen or gelatin.

In a preferred aspect, the biopolymer is a polysaccharide. The polysaccharide may be any polysaccharide, including, but not limited to, a mucopolysaccharide or a nitrogen-containing polysaccharide. In a more preferred aspect, the polysaccharide is hyaluronic acid. "Hyaluronic acid" is defined herein as an unsulphated glycosaminoglycan composed of repeating disaccharide units of N-acetylglucosamine (GlcNAc) and glucuronic acid (GlcUA) linked together by alternating beta-1,4 and beta-1,3 glycosidic bonds. Hyaluronic acid is also known as hyaluronan, hyaluronate, or HA. In another more preferred aspect, the polysaccharide is chitin. In another more preferred aspect, the polysaccharide is heparin.

In the methods of the present invention, the metabolite may be any metabolite. The metabolite may be encoded by one or more genes. The term "metabolite" encompasses both primary and secondary metabolites. Primary metabolites are products of primary or general metabolism of a strain, which are concerned, for example, with energy metabolism, growth, and structure. Secondary metabolites are products of secondary metabolism (see, for example, R.B. Herbert, *The Biosynthesis of Secondary Metabolites*, Chapman and Hall, New York, 1981).

The primary metabolite may be, but is not limited to, an amino acid, fatty acid, nucleoside, nucleotide, sugar, triglyceride, or vitamin.

The secondary metabolite may be, but is not limited to, an alkaloid, coumarin, flavonoid, polyketide, quinine, steroid, peptide, or terpene. In a preferred aspect, the secondary metabolite is an antibiotic, antifeedant, attractant, bacteriocide, fungicide, hormone, insecticide, or rodenticide.

In the methods of the present invention, the mutant of the *Aspergillus niger* strain is a recombinant strain, comprising a nucleotide sequence encoding a heterologous biological substance, *e.g.*, polypeptide, which is advantageously used in the recombinant production of the biological substance. The strain is preferably transformed with a vector comprising the nucleotide sequence encoding the heterologous biological substance followed by integration of

the vector into the chromosome. "Transformation" means introducing a vector comprising the nucleotide sequence into a host strain so that the vector is maintained as a chromosomal integrant or as a self-replicating extra-chromosomal vector. Integration is generally considered to be an advantage as the nucleotide sequence is more likely to be stably maintained in the strain. Integration of the vector into the chromosome can occur by homologous recombination, non-homologous recombination, or transposition.

The nucleotide sequence encoding a heterologous biological substance may be obtained from any prokaryotic, eukaryotic, or other source, *e.g.*, archaeabacteria. For purposes of the present invention, the term "obtained from" as used herein in connection with a given source shall mean that the biological substance is produced by the source or by a strain in which a gene from the source has been inserted.

In the methods of the present invention, the mutants of *Aspergillus niger* strains may also be used for the recombinant production of biological substances which are native to the *Aspergillus niger* strain. The native biological substance may be produced by recombinant means by, for example, placing a gene encoding the biological substance under the control of a different promoter to enhance expression of the substance, expediting its export outside the strain by use of, for example, a signal sequence, or increasing the copy number of a gene encoding the biological substance normally produced by the *Aspergillus niger* strain. Thus, the present invention also encompasses, within the scope of the term "heterologous biological substances," such recombinant production of native biological substances, to the extent that such expression involves the use of genetic elements not native to the *Aspergillus niger* strain, or use of native elements which have been manipulated to function in a manner that do not normally occur in the host strain.

The techniques used to isolate or clone a nucleotide sequence encoding a biological substance are known in the art and include isolation from genomic DNA, preparation from cDNA, or a combination thereof. The cloning of a nucleotide sequence from such genomic DNA can be effected, *e.g.*, by using the well known polymerase chain reaction (PCR). See, for example, Innis *et al.*, 1990, *PCR Protocols: A Guide to Methods and Application*, Academic Press, New York. The cloning procedures may involve excision and isolation of a desired nucleic acid fragment comprising the nucleotide sequence encoding the biological substance, insertion of the fragment into a vector molecule, and incorporation of the recombinant vector into an *Aspergillus niger* strain where multiple copies or clones of the nucleotide sequence will be replicated. The nucleotide sequence may be of genomic, cDNA, RNA, semisynthetic, synthetic origin, or any combinations thereof.

In the methods of the present invention, the biological substance may also be a fused polypeptide in which another polypeptide is fused at the N-terminus or the C-terminus of the polypeptide or fragment thereof. A fused polypeptide is produced by fusing a nucleotide sequence (or a portion thereof) encoding one polypeptide to a nucleotide sequence (or a portion thereof) encoding another polypeptide. Techniques for producing fusion polypeptides are known in the art, and include, ligating the coding sequences encoding the polypeptides so that they are in frame and expression of the fused polypeptide is under control of the same promoter(s) and terminator.

"Nucleic acid construct" is defined herein as a nucleic acid molecule, either single- or double-stranded, which is isolated from a naturally occurring gene or which has been modified to contain segments of nucleic acid which are combined and juxtaposed in a manner which would not otherwise exist in nature. The term nucleic acid construct may be synonymous with the term expression cassette when the nucleic acid construct contains all the control sequences required for expression of a coding sequence. The term "coding sequence" is defined herein as a sequence which is transcribed into mRNA and translated into a biological substance of interest when placed under the control of the below mentioned control sequences. The boundaries of the coding sequence are generally determined by an open reading frame, which usually begins with the ATG start codon or alternative start codons such as GTG and TTG. A coding sequence can include, but is not limited to, DNA, cDNA, and recombinant nucleotide sequences.

An isolated nucleotide sequence encoding a biological substance may be manipulated in a variety of ways to provide for expression of the biological substance. Manipulation of the nucleotide sequence prior to its insertion into a vector may be desirable or necessary depending on the expression vector or *Aspergillus niger* host strain. The techniques for modifying nucleotide sequences utilizing cloning methods are well known in the art.

A nucleic acid construct comprising a nucleotide sequence encoding a biological substance may be operably linked to one or more control sequences capable of directing the expression of the coding sequence in a mutant *Aspergillus niger* strain of the present invention under conditions compatible with the control sequences.

The term "control sequences" is defined herein to include all components which are necessary or advantageous for expression of the coding sequence of a nucleotide sequence. Each control sequence may be native or foreign to the nucleotide sequence encoding the biological substance. Such control sequences include, but are not limited to, a leader, a promoter, a signal sequence, and a transcription terminator. At a minimum, the control

sequences include a promoter, and transcriptional and translational stop signals. The control sequences may be provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of the control sequences with the coding region of the nucleotide sequence encoding a biological substance.

The control sequence may be an appropriate promoter sequence, which is recognized by an *Aspergillus niger* strain for expression of the nucleotide sequence. The promoter sequence contains transcription control sequences which mediate the expression of the biological substance. The promoter may be any nucleic acid sequence which shows transcriptional activity in the mutant *Aspergillus niger* strain and may be obtained from genes encoding extracellular or intracellular biological substances either homologous or heterologous to the *Aspergillus niger* strain.

Examples of suitable promoters for directing the transcription of the nucleic acid constructs in the methods of the present invention are promoters obtained from the genes for *Aspergillus oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *Aspergillus niger* neutral alpha-amylase, *Aspergillus niger* acid stable alpha-amylase, *Aspergillus niger* or *Aspergillus awamori* glucoamylase (*glaA*), *Rhizomucor miehei* lipase, *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphate isomerase, *Aspergillus nidulans* acetamidase, *Fusarium venenatum* amyloglucosidase (WO 00/56900), *Fusarium venenatum* Daria (WO 00/56900), *Fusarium venenatum* Quinn (WO 00/56900), *Fusarium oxysporum* trypsin-like protease (WO 96/00787), *Trichoderma reesei* beta-glucosidase, *Trichoderma reesei* cellobiohydrolase I, *Trichoderma reesei* endoglucanase I, *Trichoderma reesei* endoglucanase II, *Trichoderma reesei* endoglucanase III, *Trichoderma reesei* endoglucanase IV, *Trichoderma reesei* endoglucanase V, *Trichoderma reesei* xylanase I, *Trichoderma reesei* xylanase II, *Trichoderma reesei* beta-xylosidase, as well as the NA2-tpi promoter (a hybrid of the promoters from the genes for *Aspergillus niger* neutral alpha-amylase and *Aspergillus oryzae* triose phosphate isomerase); and mutant, truncated, and hybrid promoters thereof. Particularly preferred promoters are the glucoamylase, TAKA alpha-amylase, and NA2-tpi promoters.

The control sequence may also be a suitable transcription terminator sequence, a sequence recognized by an *Aspergillus niger* strain to terminate transcription. The terminator sequence is operably linked to the 3' terminus of the nucleotide sequence encoding the heterologous biological substance. Any terminator which is functional in an *Aspergillus niger* strain may be used in the present invention.

Preferred terminators are obtained from the genes encoding *Aspergillus oryzae* TAKA alpha-amylase, *Aspergillus niger* glucoamylase, *Aspergillus nidulans* anthranilate synthase,

*Aspergillus niger* alpha-glucosidase, and *Fusarium oxysporum* trypsin-like protease.

The control sequence may also be a suitable leader sequence, a nontranslated region of a mRNA which is important for translation by a *Aspergillus niger* strain. The leader sequence is operably linked to the 5' terminus of the nucleotide sequence encoding the heterologous biological substance. Any leader sequence which is functional in the *Aspergillus niger* strain may be used in the present invention.

Preferred leaders are obtained from the genes encoding *Aspergillus oryzae* TAKA alpha-amylase and *Aspergillus nidulans* triose phosphate isomerase.

The control sequence may also be a polyadenylation sequence, a sequence which is operably linked to the 3' terminus of the nucleotide sequence and which, when transcribed, is recognized by an *Aspergillus niger* strain as a signal to add polyadenosine residues to transcribed mRNA. Any polyadenylation sequence which is functional in the *Aspergillus niger* strain may be used in the present invention.

Preferred polyadenylation sequences are obtained from the genes encoding *Aspergillus oryzae* TAKA alpha-amylase, *Aspergillus niger* glucoamylase, *Aspergillus nidulans* anthranilate synthase, and *Aspergillus niger* alpha-glucosidase.

The control sequence may also be a signal peptide coding region that codes for an amino acid sequence linked to the amino terminus of the heterologous polypeptide and directs the encoded polypeptide into the strain's secretory pathway. The 5' end of the coding sequence of the nucleotide sequence may inherently contain a signal peptide coding region naturally linked in translation reading frame with the segment of the coding region which encodes the secreted polypeptide. Alternatively, the 5' end of the coding sequence may contain a signal peptide coding region which is foreign to the coding sequence. The foreign signal peptide coding region may be required where the coding sequence does not normally contain a signal peptide coding region. Alternatively, the foreign signal peptide coding region may simply replace the natural signal peptide coding region in order to obtain enhanced secretion of the polypeptide. However, any signal peptide coding region which directs the expressed heterologous polypeptide into the secretory pathway of an *Aspergillus niger* strain may be used in the present invention.

Effective signal peptide coding regions for *Aspergillus niger* host strains are the signal peptide coding regions obtained from the genes for *Aspergillus oryzae* TAKA alpha-amylase, *Aspergillus niger* neutral alpha-amylase, *Aspergillus niger* glucoamylase, *Rhizomucor miehei* aspartic proteinase, *Humicola insolens* cellulase, and *Humicola lanuginosa* lipase.

The control sequence may also be a propeptide coding region, which codes for an

amino acid sequence positioned at the amino terminus of a polypeptide. The resultant polypeptide is known as a proenzyme or propolypeptide (or a zymogen in some cases). A propolypeptide is generally inactive and can be converted to a mature, active polypeptide by catalytic or autocatalytic cleavage of the propeptide from the propolypeptide. The propeptide coding region may be obtained from the *Rhizomucor miehei* aspartic proteinase gene, or the *Myceliophthora thermophila* laccase gene (WO 95/33836).

Where both signal peptide and propeptide regions are present at the amino terminus of a polypeptide, the propeptide region is positioned next to the amino terminus of a polypeptide and the signal peptide region is positioned next to the amino terminus of the propeptide region.

The nucleic acid constructs may also comprise one or more nucleotide sequences which encode one or more factors that are advantageous for directing the expression of the heterologous biological substance, *e.g.*, a transcriptional activator (*e.g.*, a *trans*-acting factor), a chaperone, and a processing protease. Any factor that is functional in an *Aspergillus niger* strain may be used in the present invention. The nucleic acids encoding one or more of these factors are not necessarily in tandem with the nucleotide sequence encoding the heterologous biological substance.

It may also be desirable to add regulatory sequences which allow regulation of the expression of a heterologous biological substance relative to the growth of the *Aspergillus niger* strain. Examples of regulatory systems are those which cause the expression of the gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. The TAKA alpha-amylase promoter, *Aspergillus niger* glucoamylase promoter, and *Aspergillus oryzae* glucoamylase promoter may be used as regulatory sequences. Other examples of regulatory sequences are those which allow for gene amplification, *e.g.*, the metallothionein genes which are amplified with heavy metals. In these cases, the nucleotide sequence encoding the heterologous biological substance would be operably linked with the regulatory sequence.

In the methods of the present invention, a recombinant expression vector comprising a nucleotide sequence, a promoter, and transcriptional and translational stop signals may be used for the recombinant production of a polypeptide or other biological substance. The various nucleic acids and control sequences described above may be joined together to produce a recombinant expression vector which may include one or more convenient restriction sites to allow for insertion or substitution of the nucleotide sequence encoding the polypeptide or biological substance at such sites. Alternatively, the nucleotide sequence may be expressed by inserting the nucleotide sequence or a nucleic acid construct comprising the sequence into an

appropriate vector for expression. In creating the expression vector, the coding sequence is located in the vector so that the coding sequence is operably linked with the appropriate control sequences for expression, and possibly secretion.

The recombinant expression vector may be any vector which can be conveniently subjected to recombinant DNA procedures and can bring about the expression of the nucleotide sequence. The choice of the vector will typically depend on the compatibility of the vector with the *Aspergillus niger* strain into which the vector is to be introduced. The vectors may be linear or closed circular plasmids. The vector may be an autonomously replicating vector, *i.e.*, a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, *e.g.*, a plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome. The vector may contain any means for assuring self-replication. Alternatively, the vector may be one which, when introduced into the *Aspergillus niger* strain, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. The vector system may be a single vector or plasmid or two or more vectors or plasmids which together contain the total DNA to be introduced into the genome of the *Aspergillus niger* strain, or a transposon.

The vectors may be integrated into the strain's genome when introduced into an *Aspergillus niger* strain. For integration into the genome of a mutant *Aspergillus niger* strain of the present invention, the vector may rely on the nucleotide sequence encoding the heterologous biological substance or any other element of the vector for stable integration of the vector into the genome by homologous or nonhomologous recombination. Alternatively, the vector may contain additional nucleotide sequences for directing integration by homologous recombination into the genome of the *Aspergillus niger* strain. The additional nucleotide sequences enable the vector to be integrated into the genome at a precise location(s) in the chromosome(s). To increase the likelihood of integration at a precise location, the integrational elements should preferably contain a sufficient number of nucleic acids, such as 100 to 1,500 base pairs, preferably 400 to 1,500 base pairs, and most preferably 800 to 1,500 base pairs, which are highly homologous with the corresponding target sequence to enhance the probability of homologous recombination. The integrational elements may be any sequences that are homologous with the target sequence in the genome of the *Aspergillus niger*. Furthermore, the integrational elements may be non-encoding or encoding nucleotide sequences. On the other hand, the vector may be integrated into the genome of the strain by non-homologous recombination.

For autonomous replication, the vector may further comprise an origin of replication

enabling the vector to replicate autonomously in the *Aspergillus niger* in question.

The various nucleic acids and control sequences described above may be joined together to produce a recombinant expression vector which may include one or more convenient restriction sites to allow for insertion or substitution of the nucleotide sequence encoding the heterologous biological substance at such sites. Alternatively, the nucleotide sequence encoding the heterologous biological substance may be expressed by inserting the sequence or a nucleic acid construct comprising the sequence into an appropriate vector for expression. In creating the expression vector, the coding sequence is located in the vector so that the coding sequence is operably linked with the appropriate control sequences for expression, and possibly secretion.

The vectors preferably contain one or more selectable markers which permit easy selection of transformed *Aspergillus niger* strains. A selectable marker is a gene the product of which provides for biocide or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like. A selectable marker for use in an *Aspergillus niger* host strain may be selected from the group including, but not limited to, *amdS* (acetamidase), *argB* (ornithine carbamoyltransferase), *bar* (phosphinothricin acetyltransferase), *hph* (hygromycin phosphotransferase), *niaD* (nitrate reductase), *pyrG* (orotidine-5'-phosphate decarboxylase), *sC* (sulfate adenyltransferase), and *trpC* (anthranilate synthase), as well as equivalents from other species. Preferred for use in an *Aspergillus niger* strain are the *amdS* and *pyrG* genes of *Aspergillus nidulans* or *Aspergillus oryzae* and the *bar* gene of *Streptomyces hygroscopicus*.

The vectors preferably contain an element(s) that permits stable integration of the vector into the genome or autonomous replication of the vector in the strain independent of the genome of the strain.

"Introduction" means introducing a vector comprising the nucleotide sequence into an *Aspergillus niger* strain so that the vector is maintained as a chromosomal integrant or as a self-replicating extra-chromosomal vector. Integration is generally considered to be an advantage as the nucleotide sequence is more likely to be stably maintained in the strain. Integration of the vector into the chromosome occurs by homologous recombination, non-homologous recombination, or transposition.

The introduction of an expression vector into an *Aspergillus niger* host strain may involve a process consisting of protoplast formation, transformation of the protoplasts, and regeneration of the strain wall in a manner known *per se*. Suitable procedures for transformation of *Aspergillus* host strains are described in EP 238 023 and Yelton *et al.*, 1984, *Proceedings of the National Academy of Sciences USA* 81: 1470-1474.

The procedures used to ligate the elements described herein to construct the recombinant expression vectors are well known to one skilled in the art (see, *e.g.*, J. Sambrook, E.F. Fritsch, and T. Maniatus, 1989, *Molecular Cloning, A Laboratory Manual*, 2d edition, Cold Spring Harbor, New York).

In another aspect of the present invention, the mutant *Aspergillus niger* strain may further contain modifications of one or more third nucleotide sequences which encode substances that may be detrimental to the production, recovery, and/or application of the heterologous biological substance of interest. The modification reduces or eliminates expression of the one or more third nucleotide sequences resulting in a mutant strain which may produce more of the heterologous biological substance than the mutant strain without the modification of the third nucleotide sequence when cultured under identical conditions.

The third nucleotide sequence may, for example, encode an enzyme. For example, the enzyme may be an aminopeptidase, alpha-amylase, carbohydrase, carboxypeptidase, catalase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, esterase, alpha-galactosidase, beta-galactosidase, glucoamylase, alpha-glucosidase, beta-glucosidase, invertase, laccase, lipase, mannosidase, mutanase, oxidase, pectinolytic enzyme, peroxidase, phospholipase, phytase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transglutaminase, or xylanase. The third nucleotide sequence preferably encodes a proteolytic enzyme, *e.g.*, an aminopeptidase, carboxypeptidase, or protease.

The present invention also relates to methods of obtaining a mutant of a parent *Aspergillus niger* strain, comprising: (a) introducing into the *Aspergillus niger* strain a first nucleotide sequence comprising a modification of *glaA* and at least one of the genes selected from the group consisting of *asa, amyA, amyB*, *prtT* and *oah*, which are involved in the production of glucoamylase, protease, oxalic acid hydrolase, acid stable alpha-amylase, neutral alpha-amylase A, and neutral alpha-amylase B, respectively; and (b) identifying the mutant strain from step (a) comprising the modified nucleotide sequence, wherein the mutant strain is deficient in the production of glucoamylase and at least one enzyme selected from the group consisting of acid stable alpha-amylase, neutral alpha-amylase A, and neutral alpha-amylase B, protease, and oxalic acid hydrolase compared to the parent *Aspergillus niger* strain when cultivated under identical conditions.

The present invention further relates to mutants of a parent *Aspergillus niger* strain, comprising a first nucleotide sequence encoding a heterologous biological substance and one or more second nucleotide sequences comprising a modification of *glaA* and at least one of the genes selected from the group consisting of *asa, amyA, amyB*, *prtT* and *oah*, which are involved

in the production of glucoamylase, protease, oxalic acid hydrolase, acid stable alpha-amylase, neutral alpha-amylase A, and neutral alpha-amylase B, respectively, wherein the mutant strain is deficient in the production of glucoamylase and at least one enzyme selected from the group consisting of acid stable alpha-amylase, neutral alpha-amylase A, and neutral alpha-amylase B, protease, and oxalic acid hydrolase compared to the parent *Aspergillus niger* strain when cultivated under identical conditions.

The present invention is further described by the following examples which should not be construed as limiting the scope of the invention.

## Examples

All primers and oligos were supplied by MWG Biotech, Inc., High Point, NC.

DNA sequencing was conducted with an ABI 3700 Sequencing (Applied Biosystems, Inc., Foster City, CA).

### Strains

All strains are derived from *Aspergillus niger* Bo-1 (DSM 12665). *Aspergillus niger* Bo-1 comprises a mutation of the alpha-1,6-transglucosidase gene resulting in no alpha-1,6-transglucosidase activity.

### Media and Solutions

Minimal media was composed per liter of 6 g of $NaNO_3$, 0.52 g of KCl, 1.52 g of $KH_2PO_4$, 20 g of Noble Agar, 10 g of glucose, 0.5 g of $MgSO_4 \cdot 7H_2O$, and 1 ml of Cove trace elements.

Cove plates were composed per liter of 342.3 g of sucrose, 20 ml of Cove salts (50X), 10 mM acetamide, 15 mM CsCl, and 25 g of Noble agar.

50X Cove salt solution was composed per liter of 26 g of KCl, 26 g of $MgSO_4$, 76 g of $KH_2PO_4$, and 50 ml of Cove trace elements.

Cove trace elements solution was composed per liter of 0.004 g of $Na_2B_4O_7 \cdot 10H_2O$, 0.4 g of $CuSO_4 \cdot 5H_2O$, 1.2 g of $FeSO_4 \cdot 7H_2O$, 0.7 g of $MnSO_4 \cdot H_2O$, 0.8 g of $Na_2MoO_2 \cdot 2H_2O$, and 10 g of $ZnSO_4 \cdot 7H_2O$.

AMG trace metals solution was composed per liter of 14.3 g of $ZnSO_4 \cdot 7H_2O$, 2.5 g of $CuSO_4 \cdot 5H_2O$, 0.5 g of $NiCl_2$, 13.8 g of $FeSO_4$, 8.5 g of $MnSO_4$, and 3.0 g of citric acid.

YP medium was composed per liter of 10 g of yeast extract and 20 g of Bacto peptone.

STC is composed of 0.8 M sorbitol, 50 mM Tris, pH 8, and 50 mM $CaCl_2$.

SPTC was composed per liter of 40% PEG 4000, 0.8 M sorbitol, 50 mM Tris, pH 8, 50 mM $CaCl_2$.

SPC was composed per liter of 40% PEG 4000, 0.8 M sorbitol, and 50 mM $CaCl_2$ pH 4.5.

Casein plates was composed per liter of 7 g of $NaH_2PO_4 \cdot H_2O$, 0.5 g of KCl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 2 g of yeast extract, 10 g of glucose, 0.5 g of Triton X-100, 20 g of Noble agar, and 10 g of casein.

Starch azure plates were composed per liter of 0.1 g of glucose, 1 g of $KH_2PO_4$, 0.5 g of $MgSO_4$, 0.5 g of KCl, 3 g of $NaNO_3$, 0.1 g of yeast extract, 1 ml of Cove trace elements, 5 g of starch azure, 15 g of Noble agar, and 100 mM glycine pH 2.9.

**Example 1: Transformation procedure**

Twenty micrograms of each of the disruption cassettes described in the following Examples were digested with a restriction enzyme and the fragment to be used for disruption was excised and purified from a 1% agarose-50 mM Tris base-50 mM borate-0.1 mM disodium EDTA buffer (TBE) gel using a QIAEX II Gel Extraction Kit (QIAGEN, Inc., Chatsworth, CA). The total volume was brought to 20 µl in sterile glass distilled water and split between four transformations.

Protoplasts were prepared using the following protocol. Shake flasks containing 20 ml of YP medium supplemented with 5% glucose were inoculated with *Aspergillus niger* conidia at a density of *ca.* $10^6$-$10^8$ per ml. Following an overnight (15-17 hours) incubation at 34°C (200 rpm), the mycelia were collected by filtration with sterile Miracloth™ (Calbiochem, San Diego, CA) and transferred to a solution of 3-5 mg of Novozym™ 234 per ml in 10-20 ml of 1.2 M sorbitol (*Aspergillus niger* strains JRoy3, SMO110, and MBin111 through MBin114, see Examples 6-9) or 1 M $MgSO_4$ (*Aspergillus niger* strains MBin115 through MBin120, see Examples 9-12). Digestions with Novozym™ 234 were typically conducted for 30-45 minutes at 37°C with gentle shaking at 80-100 rpm. The protoplasts were filtered through sterile Miracloth™, rinsed with 1.2 M sorbitol (*Aspergillus niger* strains MBin111 through MBin114) or 2 M sorbitol (*Aspergillus niger* strains MBin115 through MBin120), and centrifuged at 3000 x *g* for 10 minutes. *Aspergillus niger* strains JRoy3, SMO110 and MBin111 through MBin114 were washed twice with 10 ml of 1.2 M sorbitol and once with 10 ml of 1.2 M sorbitol-50 mM $CaCl_2$, and then resuspended at a concentration of 3 x $10^7$–1 x $10^8$ protoplasts per ml of 1.2 M sorbitol. *Aspergillus niger* strains MBin115 through MBin120 were washed once with 30 ml of 1 M

sorbitol and once with 30 ml of STC, and then resuspended in STC:SPTC:DMSO (8:2:0.1 v/v) to achieve a concentration of $3x10^7 - 1x10^8$ protoplasts per ml. The *Aspergillus niger* protoplasts were either used directly for subsequent transformation or frozen at -80°C.

Prior to transformation of the protoplasts, selective overlay was melted and placed at 50°C. The overlay for *pyrG* selection was composed per liter of 20 ml of Cove salts, 273.8 g of sucrose, 8 g of Noble agar, 6 g of $NaNO_3$, and 1 g of NZAmine casamino acids, pH 5.5. The *pyrG* selection overlay was used for the creation of all gene disruptions. The overlay for *amdS* selection was composed per liter of 20 ml of Cove salts (50X), 273.8 g of sucrose, 8 g of Noble agar, 10 mM acetamide, and 15 mM CsCl. The *amdS* selection overlay was used when any expression plasmid was transformed.

DNA plus 5 µl of heparin (5 mg/ml of STC) was added to 100 µl of protoplasts and placed on ice for 30 minutes. *Aspergillus niger* strains prior to *Aspergillus niger* MBin115 in the lineage did not receive heparin. SPC was added (250 µl for *Aspergillus niger* strains JRoy3, SMO110 and MBin111 through MBin114 and 1 ml for the remaining strains) and mixed gently before incubation at room temperature for 30 minutes. A 10 ml volume of overlay (50°C) was added and immediately poured onto a selective plate. The selection for gene disruptions using *pyrG* as the selectable marker was minimal medium supplemented with 1 M sucrose. In generating the *Aspergillus niger* MBin111 strain, minimal medium plates composed per liter of 1 M sucrose, 1 g of 5-fluoro-orotic acid (5-FOA), and 10 mM uridine were used. Cove plates were used to select for transformants containing an expression plasmid. The plates were incubated at 34°C for 3-7 days.

**Example 2: Southern analysis**

*Aspergillus niger* mycelia were harvested from 15 mm plates containing 5 ml of YP medium supplemented with 5% glucose (and 10 mM uridine when applicable), filtered and rinsed with 10 mM Tris pH 7.4-0.1 mM EDTA pH 8 (TE) using a sidearm flask and porcelain filter, and finally placed in a microfuge tube to dry for 1 hour under a speed vacuum.

DNA was isolated using a Qiagen DNeasy Plant Mini Kit (QIAGEN, Inc., Chatsworth, CA). Five micrograms of the isolated DNA was digested for two hours (40 µl total volume, 4 U of specified restriction endonuclease/µl DNA) and electrophoresed on a 1% agarose gel using TBE buffer. The DNA was fragmented in the gel by treating with 0.25 M HCl, denatured with 1.5 M NaCl-0.5 M NaOH, and neutralized with 1.5 M NaCl-1 M Tris, pH 8, and then transferred in 20X SSC to a MSI MagnaGraph nylon transfer membrane (Micron Separations, Inc., Westborough, MA). The DNA was UV crosslinked to the membrane and prehybridized for 1

hour at 60°C in 20 ml of DIG Easy Hyb (Roche Diagnostics Corporation, Indianapolis, IN).

Probes were prepared with the PCR DIG Probe Synthesis Kit as described by the manufacturer (Roche Diagnostics Corporation, Indianapolis, IN), electrophoresed, and excised from a 1% low melt agarose gel. Prior to use, the gel was melted and the probe denatured by boiling for 10 minutes. Ten percent of the total gel volume was added to the hybridization buffer. The denatured probe was added directly to the DIG Easy Hyb buffer and an overnight hybridization at 60°C was performed. Following post hybridization washes (twice in 2X SSC, once in 0.4X SSC, 60°C, 10 minutes each), chemiluminescent detection using the DIG detection system and CPD-Star (Roche Diagnostics Corporation, Indianapolis, IN) was performed. The DIG-labeled DNA Molecular Weight Marker III (Roche Diagnostics Corporation, Indianapolis, IN) was used as a standard.

**Example 3: Construction of *Aspergillus niger* genomic lambda library**

*Aspergillus niger* Bo-1 DNA was isolated by lysis in guanidine hydrochloride according to the procedure of Wahleithner *et al.*, 1996, *Current Genetics*. 29: 395-403, followed by purification on a Qiagen Maxiprep column (QIAGEN, Inc., Chatsworth, CA) as described by manufacturer. A genomic library of *Aspergillus niger* Bo-1 was created in EMBL4 (Clonetech, Palo Alto, CA) according to the manufacturer's instructions. *Aspergillus niger* Bo-1 genomic DNA was partially digested with *Sau*3A. After digestion, the DNA was electrophoresed on a preparative low-melting-point agarose gel, and the region containing 8 to 23-kb DNA was sliced from the gel. The DNA was extracted from the gel with beta-agarase (New England Biolabs, Waltham, MA). The isolated DNA was ligated with EMBL4 arms (Clonetech, Palo Alto, CA) as described in the supplier's directions. The ligation was packaged *in vitro* with a Gigapack Gold II Packaging Kit (Stratagene, La Jolla, CA). The titer of the library was determined, and the library was amplified with *E. coli* K802 cells (American Type Culture Collection, Rockville, MD). The unamplified library was estimated to contain 26,500 independent recombinants.

**Example 4: Construction of *pyrG* cassette**

Approximately 26,500 plaques from the genomic library of *Aspergillus niger* Bo-1 contained in EMBL4 were replica plated onto nylon filters and probed with a 1.4 kb fragment from the *pyrG* gene of *Aspergillus nidulans*. Several positive clones were purified and propagated as described by the manufacturer. Phage DNA from the positive clones was isolated using a Qiagen lambda Mini Prep Kit (QIAGEN, Inc., Chatsworth, CA). Phage DNA was digested with several restriction enzymes followed by Southern analysis to identify

fragments containing the *pyrG* gene. One clone designated clone 7b contained the *Aspergillus niger pyrG* gene (SEQ ID NOs: 1 [DNA sequence] and 2 [deduced amino acid sequence]), including both the promoter and terminator sequences, on a 3.5 kb *Xba*I fragment.

The *pyrG* gene fragment was subcloned from clone 7b into pUC118 (Roche Diagnostics Corporation, Mannheim, Germany) as a 3.5 kb *Xba*I fragment resulting in pJRoy10 (Figure 1). The *pyrG* gene, including both the promoter and terminator sequences, was isolated from pJRoy10 by digestion with *Ksp*I and *Spe*I. The fragment, containing a *Ksp*I site at the 5' end and a *Spe*I site at the 3' end, was isolated using a QIAEX II Gel Extraction Kit following electrophoresis on a 1% agarose-TBE gel and purified.

A 582 bp fragment of the *pyrG* terminator sequence was PCR amplified from pJRoy10, such that *Spe*I and *Ksp*I sites were added to the 5' and 3' ends of the fragment, respectively. Primer 1 was used to create the *Spe*I site and primer 2 added the *Ksp*I site.

Primer 1: 5'-GGGACTAGTGGATCGAAGTTCTGATGGTTA-3' (SEQ ID NO: 3)

Primer 2: 5'-ATACCGCGGGTTTCAAGGATGGAGATAGGA-3' (SEQ ID NO:4)

PCR amplification was conducted in 50 µl reactions composed of 10 ng of pJRoy10 plasmid DNA, 50 pmol of each primer, 2.5 mM each of dATP, dCTP, dGTP, and dTTP, 1X PCR Buffer (Applied Biosystems, Inc., Foster City, CA) with 2.5 mM $MgCl_2$, and 2.5 units of *Taq* DNA polymerase (Roche Diagnostics Corporation, Indianapolis, IN). The reactions were performed in a RoboCycler 40 thermacycler (Stratagene, La Jolla, CA) programmed for 1 cycle at 95°C for 3 minutes; 30 cycles each at 95°C for 1 minute, 60°C for 1 minute, and 72°C for 1.5 minutes; and 1 cycle at 72°C for 5 minutes.

The 582 bp PCR product was digested with *Spe*I and *Ksp*I and used directly as described below.

Plasmid pMBin01+ (Figure 2) was constructed by ligating the *Aspergillus niger pyrG* gene fragment and the *Aspergillus niger pyrG* terminator fragment into the *Spe*I site of pBluescript SK- (Stratagene, La Jolla, CA), such that *pyrG* was flanked by 582 bp of terminator sequence on both sides. A 2696 bp *Spe*I fragment was isolated from pMBin01+ and purified using a QIAEX II Gel Extraction Kit following electrophoresis on a 1% agarose-TBE gel. Plasmid DNA was isolated using Qiagen QiaPrep8 Miniprep or Maxiprep Kits (QIAGEN, Inc., Chatsworth, CA). The 2696 bp *Spe*I fragment was then used to construct all disruption cassettes.

**Example 5: Creation of uridine auxotrophs**

Gene disruptions described in the following Examples utilized the *Aspergillus niger pyrG*

gene as a selectable marker. The *pyrG* gene encodes orotodine-5'-phosphate decarboxylase which enables an uridine auxotroph to grow without the addition of uridine. The repetitive use of *pyrG* was made possible by the addition of repeat sequence to the ends of the marker as described in Example 4. Excision of *pyrG* occurred by homologous recombination between the direct repeats upon selection on 5-FOA (d'Enfert, 1996, *Current Genetics* 30: 76-82).

As described in Example 4, the disruption cassettes contained the *pyrG* gene flanked by 582 bp of repetitive *pyrG* terminator sequence. Following gene disruption, each strain was passaged once on minimal medium containing 10 mM uridine in order to remove selection for the *pyrG* gene. Spores were collected from plates containing 10 mM uridine and transferred to minimal medium plates containing 10 mM uridine and 1 g of 5-FOA per liter. *Aspergillus niger* cells in which the *pyrG* gene was lost grow in the presence of 5-FOA while those that retain the gene convert 5-FOA to 5-fluoro-UMP, a toxic intermediate. Colonies that grew more quickly and sporulated were picked out of the lawn of slower growing non-sporulating colonies and isolated by passaging twice on minimal medium plates containing 10 mM uridine and 1 g of 5-FOA per liter and selecting for single, sporulating colonies. Southern analysis was performed as described in Example 2 to ensure that the *pyrG* gene had been excised. One copy of the *pyrG* terminator was left at the site of disruption.

**Example 6: Construction of *Aspergillus niger* SMO110 (Δ*gla*)**

The *Aspergillus niger* glucoamylase (*gla*) gene (SEQ ID NOs: 5 [DNA sequence] and 6 [deduced amino acid sequence]) was isolated from the genomic lambda library described in Example 3 as an 8 kb fragment and subcloned into pUC118 (Roche Diagnostics Corporation, Mannheim, Germany) to generate pJRoy13. A 4 kb *Spe*I fragment from pJRoy13 containing the *Aspergillus niger* glucoamylase gene and 1.8 kb of flanking DNA was inserted into pBluescriptSK+ (Stratagene, La Jolla, CA) to generate pJRoy17 (Figure 3)

A 2.3 kb *Spe*I/*Xho*I fragment containing the *pyrG* gene was isolated from pJRoy10 using a QIAEX II Gel Extraction Kit following electrophoresis on a 1% agarose-TBE gel. The restricted ends were filled in with Klenow (Roche Diagnostics Corporation, Indianapolis, IN) and the fragment was inserted into the *Bgl*II site within the glucoamylase gene coding region of pJRoy17 to create plasmid pSMO127 (Figure 4). Between two *Spe*I sites of pSMO127 was 2.3 kb of *pyrG* gene flanked by 2.2 kb and 2.3 kb of 5' and 3' glucoamylase gene sequence, respectively.

Plasmid pSMO127 was digested with *Spe*I and a 6 kb fragment consisting of the linear disruption cassette was isolated and used to transform a *pyrG* deleted strain, *Aspergillus niger*

JRoy3, using the transformation procedure described in Example 1. *Aspergillus niger* JRoy3 was obtained from *Aspergillus niger* Bo-1 using the procedure described in Example 5. Approximately 700 transformants were obtained.

A 1100 bp fragment containing the glucoamylase gene promoter was PCR amplified from the *Aspergillus niger* glucoamylase gene locus (1113 bp directly preceding the start codon) and used as a probe in Southern blot analaysis. The probe was generated with primers 3 and 4 where primer 3 hybridized to a *Spe*I site at the 5' end and primer 4 added a *Sph*I site to the 3' end.

Primer 3: 5'-ACTAGTGGCCCTGTACCCAGA-3' (SEQ ID NO: 7)

Primer 4: 5'-GCATGCATTGCTGAGGTGTAATGATG-3' (SEQ ID NO: 8)

PCR amplification of the glucoamylase gene promoter was conducted in 50 µl reactions composed of 10 ng of pJRoy17 plasmid DNA, 50 pmol of each primer, 2.5 mM each of dATP, dCTP, dGTP, and dTTP, 1X PCR Buffer with 2.5 mM $MgCl_2$, and 2.5 units of *Taq* DNA polymerase. The reactions were performed in a RoboCycler 40 thermacycler programmed for 1 cycle at 95°C for 3 minutes; 30 cycles each at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes; and 1 cycle at 72°C for 5 minutes.

The glucoamylase gene promoter probe was isolated and labeled as described in Example 2.

Genomic DNA was prepared from 200 of the 700 transformants as described in Example 2. The genomic DNA was digested with *Spe*I and then submitted to Southern analysis with the above probe using the protocol described in Example 2. A gene replacement of the disruption cassette into the glucoamylase gene locus resulted in an increase of the wild type 4 kb glucoamylase gene band to 6.3 kb, an increase due to the 2.3 kb *pyrG* gene. One such transformant was identified and designated *Aspergillus niger* SMO110.

**Example 7: Construction of *Aspergillus niger* MBin111 (Δ*pyrG*, Δ*gla*)**

The *Aspergillus niger* glucoamylase gene terminator was amplified from pJRoy17 as a 800 bp fragment with primer 5 which hybridized to the *Spe*I site at the 3' end and primer 6 that added a *Sph*I site to the 5' end.

Primer 5: 5'-GAGGTCGACGGTATCGATAAG-3' (SEQ ID NO: 9)

Primer 6: 5'-GCATGCAGATCTCGAGAATACACCGTTCCTCAG-3' (SEQ ID NO: 10)

PCR amplification of the *gla* gene terminator was conducted in 50 µl reactions composed of 10 ng of pJRoy17 plasmid DNA, 50 pmol of each primer, 2.5 mM each of dATP, dCTP, dGTP, and dTTP, 1X PCR Buffer with 2.5 mM $MgCl_2$, and 2.5 units of *Taq* DNA

polymerase. The reactions were performed in a RoboCycler 40 thermacycler programmed for 1 cycle at 95°C for 3 minutes; 30 cycles each at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes; and 1 cycle at 72°C for 5 minutes.

The 800 bp fragment containing the glucoamylase gene terminator was purified and used directly as described below.

The glucoamylase gene promoter (Example 7) and terminator PCR products were subcloned into a pCR2.1 vector using a TOPO-TA Cloning Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. A 1.1 kb *Spe*I/*Sph*I fragment containing the glucoamylase gene promoter was isolated using a QIAEX II Gel Extraction Kit following electrophoresis on a 1% agarose-TBE gel. The glucoamylase gene terminator was isolated in the same manner, however, digestion with *Spe*I/*Sph*I resulted in a 554 bp fragment due to an internal *Sph*I site. The promoter and terminator were ligated into the *Spe*I site of pBluescript SK-(Stratagene, La Jolla, CA) resulting in pMBin05 (Figure 5).

A *Spe*I fragment was removed from pMBin05 by restriction enzyme digestion and isolated using a QIAEX II Gel Extraction Kit following electrophoresis on a 1% agarose-TBE gel. The isolated fragment was transformed into *Aspergillus niger* SMO110 (Example 6) to delete the *pyrG* disrupted glucoamylase locus using the transformation procedure described in Example 1. Prior to plating the transformation on 5-FOA to select for the *pyrG* minus phenotype (see Example 5), an outgrowth was performed to allow more time for recombination prior to selection. The outgrowth was conducted in 5 ml of YP medium supplemented with 5% glucose, 0.9 M sucrose, and 10 mM uridine for 24 hours at 37°C and 100 rpm.

Nine transformants were obtained and one maintained the *pyrG*- phenotype when transferred to selective media described in Example 5. The transformant maintaining the *pyrG*- phenotype was designated *Aspergillus niger* MBin111.

Probes were generated to the *Aspergillus niger* glucoamylase and *pyrG* genes. Primers 3 and 5 above were used to PCR amplify the *gla* gene (including promoter and terminator) from pJRoy17 and primers 1 and 2 (see Example 4) were used to amplify the *pyrG* terminator sequence from pJRoy10 using the same procedure described in Example 4. The probes were isolated and labeled as described in Example 2.

Genomic DNA was isolated from *Aspergillus niger* strains JRoy3, SMO110, and MBin111 as described in Example 2, digested with *Spe*I, and probed with the *Aspergillus niger* glucoamylase gene according to the protocol described in Example 2 for Southern analysis. A 4 kb band representing the undisrupted *gla* gene locus was observed in *Aspergillus niger* JRoy3 and a 6.3 kb band, due to the insertion of the disruption cassette, was obtained from *Aspergillus*

*niger* SMO110. No hybridization was detected with genomic DNA from *Aspergillus niger* MBin111, indicating that the glucoamylase gene had been deleted. Moreover, DNA digested with *Spe*I was probed with the *pyrG* terminator sequence and again no hybridization was observed in the *Aspergillus niger* MBin111 strain, but *Aspergillus niger* SMO110 maintained the 6.3 kb band. These results indicated that the entire glucoamylase gene locus and *pyrG* gene were deleted in *Aspergillus niger* MBin111.

**Example 8: Construction of *Aspergillus niger* MBin112 (Δ*asa*, Δ*pyrG*, Δ*gla*)**

A portion of the *Aspergillus niger* acid stable alpha-amylase gene (*asa*) was isolated and cloned into pUC19 (Roche Diagnostics Corporation, Mannheim, Germany) as described in U.S. Patent No. 5,252,726. A 101 bp fragment, 346 bp upstream of the start codon of the portion of the acid stable alpha-amylase gene, was excised from pUC19 containing the portion of the acid stable alpha-amylase gene by digestion with *Hpa*I and the *Spe*I fragment from pMBin01 (Example 4) was inserted into this site by blunt end ligation to create pMBin04+ (Figure 6). A double digest of pMBin04+ was performed with *Sma*I and *Spe*I and a 4237 bp *Sma*I/*Spe*I fragment was isolated using a QIAEX II Gel Extraction Kit following electrophoresis on a 1% agarose-TBE gel. The 4237 bp *Sma*I/*Spe*I fragment consisted of the 5' end of acid stable alpha-amylase gene, the *pyrG* terminator, the entire *pyrG* gene (including the terminator), and the 3' end of the acid stable alpha-amylase gene.

*Aspergillus niger* strain MBin111 was transformed with the *Sma*I/*Spe*I fragment from pMBin04+ using the transformation procedure described in Example 1. Totally, 160 transformants were obtained on minimal medium. The transformants were then transferred to starch azure plates to screen for those lacking acid stable alpha-amylase activity. Sixteen transformants produced little or no clearing zones and were single colony isolated twice on minimal medium supplemented with 10 mM uridine.

A 522 bp fragment was PCR amplified from the acid stable alpha-amylase gene locus and used as a probe in Southern blot analysis. The probe was generated with primers 7 and 8.

Primer 7: 5'-CTCATTGGCCGAAACTCCGAT-3' (SEQ ID NO: 11)

Primer 8: 5'-AGCAGACGATGTCCTGAGCTG-3' (SEQ ID NO: 12)

PCR amplification of the 522 bp fragment was conducted in 50 µl reactions composed of 10 ng of pUC19/HW360 plasmid DNA, 50 pmol of each primer, 2.5 mM each of dATP, dCTP, dGTP, and dTTP, 1X PCR Buffer with 2.5 mM $MgCl_2$, and 2.5 units of *Taq* DNA polymerase. The reactions were performed in a RoboCycler 40 thermacycler programmed for 1 cycle at 95°C for 3 minutes; 30 cycles each at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute;

and 1 cycle at 72°C for 5 minutes.

The 522 bp probe was isolated and labeled as described in Example 2.

Genomic DNA was isolated as described in Example 2 from the 16 transformants and untransformed *Aspergillus niger* strain MBin111 as a control. The genomic DNA was then digested with *Xho*I and *Spe*I and submitted to Southern hybridization as described in Example 2 using the probe above. The intact acid stable alpha-amylase gene locus was visualized as a 2.3 kb band and the disrupted locus was 5.3 kb in size. This size difference is due to the insertion of the 3 kb pMBin01+ *SpeI* fragment described in Example 4. Five transformants containing an acid stable alpha-amylase gene disruption were obtained and one was designated *Aspergillus niger* MBin112. The loop-out of the disruption cassette, resulting in *Aspergillus niger* strain MBin113, left behind the *pyrG* terminator and created a 2.8 kb band. The loop-out was performed as described in Example 5 and resulted in *Aspergillus niger* MBin113.

**Example 9: Construction of *Aspergillus niger* MBin114 (Δ*prtT*, Δ*asa*, Δ*pyrG*, Δ*gla*)**

The *Aspergillus niger prtT* gene (SEQ ID NOs: 13 [DNA sequence] and 14 [deduced amino acid sequence]) was constructed (pMBin09, Figure 7) using two overlapping clones, NcE 1.4 and ClE 1.8, described in WO 00/20596. NcE 1.4, ClE 1.8, and pZeRO-2 (Invitrogen, Carlsbad, CA) were digested with *Pst*I, generating *Pst*I sites at the 5' and 3' ends of the clones respectively and linearizing pZeRO-2 at the multiple cloning site. Utilizing a *Ssp*I site in a shared region of both *prtT* clones, a three way ligation was performed by ligating the *Pst*I/*Ssp*I clone fragments into pZeRO-2 at the *Pst*I site, resulting in pMBin09.

A 233 bp deletion of the *prtT* coding sequence was first made by digestion of pMBin09 with *Bst*1107I/*Ssp*I and the pMBin01 *Spe*I fragment described in Example 4 was inserted as a blunt fragment into the digested pMBin09 to create pMBin10 (Figure 8). The *prtT* disruption was performed using the *Dra*III/*Nhe*I fragment from pMBin10 which was isolated using a QIAEX II Gel Extraction Kit following electrophoresis on a 1% agarose-TBE gel.

*Aspergillus niger* MBin113 was transformed with the *Dra*III/*Nhe*I fragment from pMBin10 using the transformation procedure described in Example 1. One hundred and two transformants were screened on casein plates. Nine transformants showed little or no clearing and were single colony isolated twice on minimal medium supplemented with 10 mM uridine.

A 232 bp fragment of the *prtT* coding sequence was PCR amplified from the *prtT* locus in pMBin10 and used as a probe in Southern blot analysis. The fragment was generated using primers 9 and 10.

Primer 9: 5'-TGTGATTGAGGTGATTGGCG-3' (SEQ ID NO: 15)

Primer 10: 5'-TCAGCCACACCTGCAAAGGC-3' (SEQ ID NO: 16)

PCR amplification was conducted in 50 µl reactions composed of 10 ng of pMBin10 plasmid DNA, 50 pmol of each primer, 2.5 mM each of dATP, dCTP, dGTP, and dTTP, 1X PCR Buffer with 2.5 mM $MgCl_2$, and 2.5 units of *Taq* DNA polymerase. The reactions were performed in a RoboCycler 40 thermacycler programmed for 1 cycle at 95°C for 3 minutes; 30 cycles each at 95°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute; and 1 cycle at 72°C for 5 minutes.

The probe was isolated and labeled as described in Example 2 and contained 232 bp of the *prtT* coding sequence downstream of the disruption.

Genomic DNA was isolated as described in Example 2 from the 9 transformants, as well as *Aspergillus niger* Bo-1 and *Aspergillus niger* MBin112 as controls, and submitted to Southern analysis as described in Example 2. The genomic DNA was digested with *Pst*I and a 2.5 kb band, corresponding to the undisrupted *prtT* gene, was observed in the control strains. A band at 1.3 kb, corresponding to a *prtT* gene disruption, was observed when the probe hybridized to a *Pst*I fragment containing 132 bp of the *pyrG* terminator and 1198 bp of the *prtT* gene. One disruptant was chosen and designated *Aspergillus niger* MBin114. The *pyrG* gene was looped out as described in Example 5 resulting in *Aspergillus niger* MBin115.

**Example 10: Construction of *Aspergillus niger* MBin116 (Δ*amyB*, Δ*prtT*, Δ*asa*, Δ*pyrG*, Δ*gla*)**

The *Aspergillus niger* neutral alpha-amylase genes, *amyA* and *amyB*, were cloned as disclosed in U.S. Patent No. 5,252,726 (NA1=*amyA* and NA2=*amyB*).

A 2.6 kb fragment of the *Aspergillus niger* neutral alpha-amylase gene (*amyB*) (SEQ ID NOs: 17 [DNA sequence] and 18 [deduced amino acid sequence]) was isolated from pTaka17 (U.S. Patent No. 5,536,661) by *Eco*RI/*Bgl*II digestion and isolated using a QIAEX II Gel Extraction Kit following electrophoresis on a 1% agarose-TBE gel. The 2.6 kb fragment was inserted into the *Eco*RI/*Bam*HI site of pZero2.0 (Invitrogen, Carlsbad, CA) to create pMBin02 (Figure 9). A 298 bp deletion that removed 186 bp from the fifth exon and 52 bp from the sixth exon of the homologous *amyB* gene was made in pMBin02 by *Pme*I/*Sma*I digestion and the pMBin01 2696 bp *Spe*I fragment (described in Example 4) was inserted by blunt end ligation to create pMBin03 (Figure 10).

*Aspergillus niger* MBin115 was transformed using the protocol described in Example 1 with an *Eco*RI/*Avr*II fragment isolated from pMBin03. One hundred and ninety two

transformants were obtained and transferred to starch azure plates as described in Example 8 with the following changes: the starch azure plates lacked glycine and the pH was at 5. Eight transformants showed reduced clearing zones and were single colony isolated twice on minimal medium supplemented with 10 mM uridine.

A probe with a sequence corresponding to 295 bp of the *Aspergillus niger amyA or amyB* coding sequence, 450 bp downstream of the ATG site (the *amyA* and *amyB* sequences are identical in this region), was generated by PCR amplification using primers 11 and 12.

Primer 11: 5'-GGCAGCAGGATATGTAAGTCG-3' (SEQ ID NO: 19)

Primer 12: 5'-CACTGTAATCGACTGAGCTAC-3' (SEQ ID NO: 20)

PCR amplification was conducted in 50 µl reactions composed of 10 ng of pMBin03 plasmid DNA, 50 pmol of each primer, 2.5 mM each of dATP, dCTP, dGTP, and dTTP, 1X PCR Buffer with 2.5 mM $MgCl_2$, and 2.5 units of *Taq* DNA polymerase. The reactions were performed in a RoboCycler 40 thermacycler programmed for 1 cycle at 95°C for 3 minutes; 30 cycles each at 95°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute; and 1 cycle at 72°C for 5 minutes.

The probe was isolated and labeled as described in Example 2. Genomic DNA was isolated as described in Example 2 from the 8 transformants and untransformed *Aspergillus niger* MBin115 as a control and digested with *Eco*RI and *Bsp*LU11I. The digested genomic DNA was submitted to Southern analysis using the procedure described in Example 2. There was an *Eco*RI site 616 bp upstream of the start codon and a *Bsp*Lu11I site 99 bp downstream of the stop codon. The wildtype *Aspergillus niger* strain Bo-1 *amyB* gene band was 2659 bp. Disruption of the *amyB* gene resulted in the disappearance of the 2659 bp band and the appearance of a band at 5359 bp due to the insertion of the pMBin01 *Spe*I fragment.

One transformant contained a clean disruption and was designated *Aspergillus niger* MBin116. The *pyrG* gene was excised from *Aspergillus niger* MBin116 as described in Example 5 and the strain was designated *Aspergillus niger* MBin117.

**Example 11: Construction of *Aspergillus niger* MBin118 (Δ*amyA*, Δ*amyB*, Δ*prtT*, Δ*asa*, Δ*pyrG*, Δ*gla*)**

Since the *Aspergillus niger amyA* gene sequence is essentially identical to *amyB*, except at the 3' end (Korman *et al.*, 1990, *Current Genetics* 17: 203-212), the disruption construct and protocol used in Example 10 was applied. *Aspergillus niger* MBin117 was transformed according the protocol described in Example 1 with the *Eco*RI/*Avr*II fragment from pMBin03 in order to disrupt the *amyA* gene (SEQ ID NOs: 21 [DNA sequence] and 22 [deduced amino acid

sequence]).

Three hundred and fifty six transformants were obtained and transferred to starch azure plates as described in Example 10. Four transformants producing no clearing zones on the starch azure plates were single colony isolated twice on minimal medium supplemented with 10 mM uridine.

Genomic DNA was isolated from the 4 transformants and *Aspergillus niger* MBin117 as a control and submitted to Southern analysis using the procedures described in Example 2. The genomic DNA was digested with *Eco*RI and *Bsp*LU11I and probed as described in Example 10. A 2.7 kb band corresponding to the *amyB* gene and a slightly larger band representing the *amyA* gene were present in the wild type *Aspergillus niger* Bo-1 strain. The exact size of the *amyA* band was not known since *Bsp*LU11I cuts at an unknown site downstream of the *amyA* gene. In one of the transformants analyzed, a band corresponding to the *amyA* gene was no longer visible with the probe indicating that a deletion of the *amyA* gene encompassing the location of the probe had occurred. The transformant was designated *Aspergillus niger* MBin118. The *pyrG* gene was excised from *Aspergillus niger* MBin118 as described in Example 5 and the strain was designated *Aspergillus niger* MBin119.

**Example 12: Construction of *Aspergillus niger* MBin120 (Δ*oxa*, Δ*amyA*, Δ*amyB*, Δ*prtT*, Δ*asa*, Δ*pyrG*, Δ*gla*)**

An *Aspergillus niger* oxalic acid hydrolase (*oah*) gene (SEQ ID NOs: 23 [DNA sequence] and 24 [deduced amino acid sequence]) was cloned according to the procedure described in WO 00/50576. Plasmid pHP1 was constructed as described in WO 00/50576.

A 285 bp deletion, which included 156 bp of the promoter and 129 bp of the oxalic acid hydrolase gene coding sequence, was removed by digesting pHP1 with *Bst*EII. The pMBin01 *Spe*I fragment described in Example 4 was blunt end ligated into this site to create pMBin08 (Figure 11). Plasmid pMBin08 was digested with *Not*I and a fragment of 7155 bp was isolated using a QIAEX II Gel Extraction Kit following electrophoresis on a 1% agarose-TBE gel. The *Not*I fragment from pMBin08 was used to disrupt the oxalic acid hydrolase gene in *Aspergillus niger* MBin119.

*Aspergillus niger* MBin119 was transformed with the *Not*I fragment from pMBin08 using the transformation procedure described in Example 1. Forty-nine transformants were obtained and screened for oxalate production using a Sigma Oxalate Kit (number 591, Sigma Diagnostics, St. Louis, MO). The transformants were cultivated in shake flasks by inoculating conidia of transformants at a density of *ca.* $10^4$ per ml into 125 ml shake flasks containing 20 ml

of YP medium supplemented with 5% glucose. The shake flasks were incubated 3 to 6 days at 37°C and 200 rpm. Samples of 5 µl of the shake flask cultures were removed at day 3 and centrifuged to produce supernatants for enzyme assay. The day 3 supernatants were added to wells in a 96 well plate followed by the oxalate kit reagents as specified by the manufacturer, but at 1/10th of the volume. Production of oxalate was measured spectophotometrically at 590 nm. One transformant produced no detectable oxalate and was single colony isolated twice on minimal medium supplemented with 10 mM uridine.

A fragment comprising 579 bp of sequence from within the oxalic acid hydrolase gene (404 bp downstream of the start codon) was PCR amplified for use as a probe in Southern blot analysis using primers 13 and 14.

Primer 13: 5'-CTACGACATGAAGACCAACGC-3' (SEQ ID NO: 25)

Primer 14: 5'-GCACCGTTCTCCACCATGTTG-3' (SEQ ID NO: 26)

PCR amplification was conducted in 50 µl reactions composed of 10 ng of pMBin08 plasmid DNA, 50 pmol of each primer, 2.5 mM each of dATP, dCTP, dGTP, and dTTP, 1X PCR Buffer with 2.5 mM $MgCl_2$, and 2.5 units of *Taq* DNA polymerase. The reactions were performed in a RoboCycler 40 thermacycler programmed for 1 cycle at 95°C for 3 minutes; 30 cycles each at 95°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute; and 1 cycle at 72°C for 5 minutes.

The probe was isolated and labeled as described in Example 2. Genomic DNA from the transformant, as well as *Aspergillus niger* Bo-1 and *Aspergillus niger* MBin118 as controls, was isolated as described in Example 2 and digested with *Nde*I and *Ssp*I. Southern analysis of *Aspergillus niger* control strains Bo-1 and MBin118 with the probe above revealed a 2.5 kb band corresponding to the undisrupted oxalic acid hydrolase gene. The transformant had a 4.9 kb band consistent with the insertion of the disruption cassette at the oxalic acid hydrolase gene locus. The transformant was designated *Aspergillus niger* MBin120.

**Example 13: Expression analysis of *Aspergillus niger* general host strains**

The ability of the general host *Aspergillus niger* strains to produce glucoamylase, acid stable alpha-amylase, neutral alpha-amylase, and protease was evaluated by cultivating the strains in shake flasks and/or fermentors. *Aspergillus niger* Bo-1 was run as a control.

Conidia of the *Aspergillus niger* strains at a density of *ca.* $10^4$ per ml were inoculated into 125 ml shake flasks containing 20 ml of YP medium supplemented with 5% glucose. The shake flasks were incubated 3 to 6 days at 37°C and 200 rpm. Samples of the shake flask cultures were removed at days 3 - 6 and centrifuged to produce supernatants for enzyme assay.

*Aspergillus niger* strains were also inoculated into 2 liter fermentors containing 1.8 liters of medium composed per liter of 2 g of $MgSO_4 \cdot 7H_2O$, 2 g of $KH_2PO_4$, 2 g of citric acid, 2 g of $K_2SO_4$, 0.5 ml of AMG trace metals solution, 300 g of high maltose syrup, 1.8 g of $CaCl_2 \cdot 2H_2O$, and 1.8 ml of pluronic acid. The fermentation medium was fed with a medium composed per liter of 50 g of urea and 5 ml of pluronic acid. The conditions of the fermentations were 34°C at pH 4.5 +/-0.05, 1.0 vvm aeration, and 1000 rpm for 8 days. Samples of the fermentations were removed at days 1 - 8 and centrifuged to produce supernatants for enzyme assay.

Glucoamylase activity was measured at 25°C in 0.1 M sodium acetate at pH 4.3 using maltose as the substrate. Glucose was measured using the Sigma Trinder color reagent (Sigma reagent kit 315-100, Sigma Chemical Co., St. Louis, MO) at 490 nm according to the manufacturer's instructions. AMG™ (Novozymes A/S, Bagsværd, Denmark; batch 7-195) was used as a standard with glucoamylase activity measured in AGU/ml.

*Aspergillus niger* SMO110 was determined to produce no detectable glucoamylase activity (less than 0.5 AGU/ml in day 4 shake flask samples). *Aspergillus niger* MBin111 was determined to produce no detectable glucoamylase activity (less than 0.5 AGU/ml in day 4 shake flask or fermentation samples).

Acid stable and neutral alpha-amylase activity was measured at pH 4.5 and pH 7.0, respectively, using a Sigma alpha-amylase substrate (Sigma Kit # 577, Sigma Chemical Co., St. Louis, MO) at 30°C. Detection was at 405 nm. Fungamyl™ was used as a standard and activity was reported in FAU/ml.

Acid stable alpha-amylase activity was found to be barely detectable with *Aspergillus niger* MBin113, MBin116, and MBin118 (>0.1 FAU/ml in both day 3 shake flask or fermentation samples) compared to *Aspergillus niger* Bo-1 (51 FAU/ml in day 5 fermentation samples). Neutral alpha-amylase activity was substantially reduced with *Aspergillus niger* MBin114 (not detectable from day 3 shake flask samples and 5.7 FAU/ml in day 5 fermentation samples) and barely detectable with *Aspergillus niger* MBin118 (0.5 FAU/ml in day 5 fermentation samples) compared to *Aspergillus niger* Bo-1 in fermentation samples.

General protease activity was determined using FITC-casein as substrate (Sigma Chemical Co., St. Loius, MO). The assay was conducted by mixing 40 µl of FITC-casein substrate (stock solution: 1:1 with 0.1 M potassium phosphate pH 6.0 or 0.1 M sodium citrate pH 5.0) with 10 µl of culture sample diluted appropriately in 0.1 M potassium phosphate pH 6.0 or 0.1 M sodium citrate pH 5.0 and incubating the solution for 1 hour at 37°C. After the 1 hour incubation, the reaction was quenched with 150 µl of 5% trichloroacetic acid and incubated in a

cold room for 1 hour. The quenched reaction was transferred to an Eppendorf tube and centrifuged for 10 minutes. A 10 µl aliquot of the supernatant was transferred to a test tube containing 1 ml of 0.5 M borate pH 9.0 and mixed. A 200 µl aliquot of the solution was transferred to a black "U" bottom 96 well plate (ThermoLabsystems, Franklin, MA). Fluorescence was measured using a Fluorolite 1000 instrument (ThermoLabsystems, Franklin, MA) using reference channel 3 and a setting of 1176. Activity was measured in protease fluorescent units.

With the deletion of the *prtT* gene in *Aspergillus niger* MBin114, total protease activity dropped to about 20% of *Aspergillus niger* Bo-1. Day 6 fermentation samples of MBin114 had a protease activity of 692 while Bo-1 was at 3953 fluorescent units/ml.

**Example 14: Expression of *Candida antarctica* lipase B in *Aspergillus niger* MBin114, MBin118 and MBin120**

The *Candida antarctica* lipase B gene (SEQ ID NOs: 27 [DNA sequence] and 28 [deduced amino acid sequence]) was cloned as described in U.S. Patent No. 6,020,180. Plasmid pMT1335 containing the lipase B gene was constructed as described by Hoegh *et al.*, in *Can. J. Bot.* 73 (Suppl.1): S869-S875 (1995). Plasmid pTOC90 containing an *Aspergillus nidulans amdS* gene was constructed as described in WO 91/17243. Plasmids pMT1335 and pTOC90 were co-transformed into *Aspergillus niger* MBin114 according to the protocol described in Example 1 and transformants were selected on acetamide.

Thirty transformants were isolated by streaking to acetamide plates. Conidia were collected from the transformants and used to inoculate shake flasks as described in Example 13. Samples of the shake flask cultures were removed at days 3 - 6 and centrifuged to produce supernatants for enzyme assay.

In order to assess the effect disruption of the *prtT* gene had on the total level of protease activity and the yield of *Candida antarctica* lipase B (CLB), both protease and lipase B activities were determined. Several transformants produced lipase B and the highest producer was evaluated by fermentation.

*Aspergillus niger* MBin114 and *Aspergillus niger* Bo-1, as a control, were cultivated in 2 liter fermentors as described in Example 13.

General protease activity was measured as described in Example 9.

Lipase B assays were performed at pH 7 with a p-nitrophenyl butyrate (Sigma Chemical Co., St. Louis, MO) as substrate. Culture supernatants were diluted as appropriate in 0.1 M MOPS-4 mM $CaCl_2$ pH 7.0. A 100 µl aliquot of a culture supernatant was added to 100 µl of p-

nitrophenyl butyrate substrate solution in wells of a 96 well microplate. The p-nitrophenyl butyrate substrate solution was composed of 10 µl of p-nitrophenyl butyrate, 990 µl of DMSO, and 4 ml of 0.1 M MOPS-4 mM $CaCl_2$ pH 7.0. Lipase activity was measured spectrophotometrically at 405 nm using a *Candida antarctica* lipase B standard (Novozymes Japan Ltd., Chiba-shi, Japan) to calculate LU/ml.

Figures 14 and 15 show the results of these assays. Total protease activity dropped to about 20% of wildtype (see Example 13, Figure 12) and lipase B activity rose steadily throughout the fermentation (Figure 13).

**Example 15: Expression of *Scytalidium thermophilum* catalase in *Aspergillus niger* MBin114, MBin118 and MBin120**

The *Scytalidium thermophilum* catalase gene (SEQ ID NOs: 29 [DNA sequence] and 30 [deduced amino acid sequence]) was cloned as described in U.S. Patent No. 5,646,025. Plasmid pDM153 containing the catalase gene was constructed as described in U.S. Patent No. 5,646,025. Plasmid pDM153 was transformed into *Aspergillus niger* strains MBin114, MBin118, and MBin120 according to the protocol described in Example 1.

Forty transformants were selected and cultivated in 24 well plates containing 1.5 ml of a 1:4 dilution of M400 medium. The plates were incubated for 90 hours at 34°C and 125 rpm. Samples for assay were removed at 90 hours. The three transformants that produced the highest level of catalase activity were evaluated in fermentors.

Catalase activity was measured at 25°C in 10 mM phosphate pH 7 buffer containing 18.2 µl of a stock hydrogen peroxide solution. The stock hydrogen peroxide solution was composed of 30% hydrogen peroxide per 10 ml of 10 mM potassium phosphate pH 7. A 25 µl aliquot of culture supernatant was added to 25 µl of hydrogen peroxide stock solution in wells of a 96 well microplate. Following 5 minutes of incubation, 200 µl of titanium reagent was added and the absorbance was read at 405 nm. The titanium reagent was composed of 1.0 g of titanium oxide and 10 g of $K_2SO_4$, which was digested for 2-3 hours with 150 ml of concentrated $H_2SO_4$ at 180-220°C, allowed to cool, and then diluted with 1.5 liters of deionized water. The catalase activity was measured spectrophotometrically at 405 nm using Catazyme™ (Novozymes A/S, Bagsværd, Denmark, batch 31-2197) as a standard and reported in KCIU/ml.

*Aspergillus niger* strains MBin114, MBin118, and MBin120 were cultivated in 2 liter fermentors as described in Example 13.

Figure 15 shows a comparison of *Scytalidium thermophilum* catalase production in

*Aspergillus niger* general host strains MBin114, MBin118 and MBin120. No obvious change in enzyme production was observed in any of the strains tested.

The invention described and claimed herein is not to be limited in scope by the specific aspects herein disclosed, since these aspects are intended as illustrations of several aspects of the invention. Any equivalent aspects are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. In the case of conflict, the present disclosure including definitions will control.

Various references are cited herein, the disclosures of which are incorporated by reference in their entireties.